MATERNAL TASTE FUNCTION AND THE PROGRAMMING OF UNHEALTHY TASTE RESPONSES IN OFFSPRING

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MATERNAL TASTE FUNCTION AND THE PROGRAMMING OF UNHEALTHY TASTE RESPONSES IN OFFSPRING

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Despite the importance of the maternal diet to supply adequate nutrition to the developing fetus, little is known about how the maternal taste system changes during pregnancy; and further if maternal over-nutrition has any long-term impact on the taste system of the offspring in adulthood. Given that more than half of women at childbearing age are considered to be overweight or obese, it is vital to understand how taste can change during pregnancy, and further, to study offspring taste development in the context of maternal obesity.

The following experiments were designed to investigate the taste system at the level of behavior, morphology, and gene expression in pregnant mice. In brief-access taste testing with partially food and water restricted mice, licking responses to sucrose decreased during pregnancy and returned to baseline postpartum. Taste bud morphology was unchanged across pregnancy, however taste receptor expression levels were altered across multiple time points during gestation and postpartum. The results indicate that the physiological changes induced by pregnancy can influence taste gene expression, and that interventions focused on the taste bud represent a useful strategy to enhance offspring fitness through maternal intake.

The long-term effects of gestational obesity were studied by providing maternal mice with *ad libitum* high-fat diet throughout pregnancy, weaning their offspring onto

normal chow, and studying the taste behavior of the offspring as adults. The adult offspring of mice fed a high fat diet showed enhanced licking responses to sucrose in brief-access testing. Despite only having contact with the high-fat diet *in utero* and through lactation, this behavior was associated with an increase in sweet receptor expression, and an increase in intake for sweet solutions and the high-fat diet. It is possible that this altered taste system may arise from early fetal programming.

In summation, these findings highlight the importance of studying maternal diet and the long-term impacts of maternal obesity on the offspring taste system. Greater understanding of how the maternal diet contributes to the development of the offspring is critical for finding solutions to overcome diseases related to over-nutrition, and to promote healthy eating habits for children who struggle with obesity and diabetes.

BIOGRAPHICAL SKETCH

Ezen Choo was born in Sydney, Australia and raised in California where she earned a B.S. in Environmental Toxicology at the University of California, Davis. As a President's Undergraduate Fellow, she used yeast as a model organism to study the effects of oxidative stress on protein folding. After graduating, she worked in Silicon Valley at a start-up working on a continuous glucose-monitoring device. She went on to work for a nonprofit organization in science education research focusing on virtual game-based teaching and assessment.

Ezen matriculated into the Ph.D. Pharmacology program at Cornell University in 2012, where she was able to combine her interests in food as medicine, physiology, and neurobiology in the lab of Dr. Robin Dando. Her dissertation focuses on novel ways to promote healthy eating by studying the taste system during pregnancy with two main branches – the mother and the offspring.

Over the years at Cornell, Ezen has been a Scholarship of Teaching and Learning Fellow (2015), a Vertebrate Genomics Scholar (2016), and an inductee into the Bouchet Graduate Honor Society (2017) in recognition of her scholarly achievements, personal excellence, and for serving as an example of scholarship, leadership, character, and service and advocacy for students. Ezen has won awards for on-campus research presentations as well as travel awards to present at national conferences including the Association of Chemoreception Sciences and the American Society for Pharmacology and Experimental Therapeutics.

Ezen served three years on the Coordinating Committee for the Graduate Research Student Outreach Program (GRASSHOPR) and taught mini-courses on taste research at Ithaca High School. She also served as a student ambassador for the Biomedical & Biological Sciences program and was the co-founder and President of the Biomedical and Biological Sciences Graduate Student Society (BBSGSS).

To my beloved family and friends

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CHAPTER 1

Background and Introduction

The importance of the maternal diet during pregnancy

The gustatory system represents a novel target for regulating food intake and eating behavior. There is a dire need for interventions to motivate women to adopt healthy eating habits during pregnancy (Trout and Effinger 2012), especially given the increasing evidence that those habits may have persistent, lifelong effects in their offspring. However, much of our knowledge of taste modulation during human pregnancy remains inconsistent. Studies have focused particularly on sweet taste, suggesting an increase in preference during pregnancy (Worthington-Roberts et al 1989; Pope et al 1992; Bayley et al 2002). Others have implicated a general decline in gustatory function during early to mid pregnancy (Duffy et al 1998; Kölble et al 2001; Kuga et al 2002; Ochsenbein-Kölble et al 2005). Although studies looked into the mechanisms involved in the etiology of food cravings in other settings (such as in pica, menstruation, hypertension, etc.; reviewed by Orloff and Hormes 2014), the role of the taste buds themselves in food cravings during pregnancy remains relatively uncharted. Understanding how taste influences the maternal diet during pregnancy is critical as these experiences set the stage for later food choices and establishing life-long food habits in the offspring.

The importance of the maternal diet on offspring health

The propensity to develop weight related diseases are acquired early in life. The nutritional environment during the critical developmental period *in utero* has remarkable

impact on the risk of the fetus for developing metabolic syndrome (Lakshmy 2013; Brenseke et al 2013; Mischke and Plosch 2013), obesity (Shankar et al 2008, Treesukosol et al 2014), and cardiovascular dysfunction (Samuelsson et al 2013). Gestational weight gain is also a strong predictor for future over-weight or obese status in children and adolescents (Orloff and Hormes 2014). Interestingly, earlier studies found dietary fat intake as a strong predictor of obesity in adult women who were once lean, but only if they had a family history of obesity. This suggests that a preference for palatable foods precedes the development of obesity (Reed et al 1997).

The earliest fetal exposure to flavor occurs first *in utero* via the amniotic fluid and then later through the breast or formula milk. The sensory environment changes as a function of the maternal food choices and dietary flavors are transmitted through the amniotic fluid (Mennella et al 1995). There is evidence to suggest that these early exposures can later impact food preferences (Cooke and Fildes 2011), but the extent to which the compounds in the mother's diet can be transmitted to the offspring and by what mechanisms remains to be investigated. Considering that many diseases that plague developed and developing societies involve excess food consumption, an understanding of the factors that influence food choice, particularly an understanding of factors during the developmental window, are essential for enhancing the health of the offspring and adult.

Taste structure and function

From an evolutionary perspective, a child eating foods their mother ate would ensure familiar safe foods are being consumed. Fetal taste buds mature in humans *in utero* by 13 to 15 weeks of gestation, with taste receptor cells appearing at 16 weeks

(Blackburn 2003; Blackburn 2007). Lingual taste buds are found within gustatory papillae and are similarly distributed over the tongue of humans and other mammals. Gustatory papillae include the circumvallate, foliate, and fungiform. Taste buds are clusters of 50-100 specialized cells equipped with the machinery to recognize the five basic tastes: sweet, salty, sour, bitter, and umami. The taste receptor type 1 family includes the GPCRs T1R1, T1R2, and T1R3. The T1R1+T1R3 dimer composes the receptor for umami (Zhao et al 2003). Sweet compounds, including artificial sweeteners (Zhao et al 2003), activate T1R2+T1R3 (Figure 1). Tas2R receptors (25 variations in humans) allow the detection of a broad spectrum of bitter compounds (Mueller et al 2005). Ion channels are thought to recognize protons in salty (αENaC) and sour (possibly related to PKD2L1 and PKD1L3) tastes. See review by Chaudhari and Roper 2010 for further details.

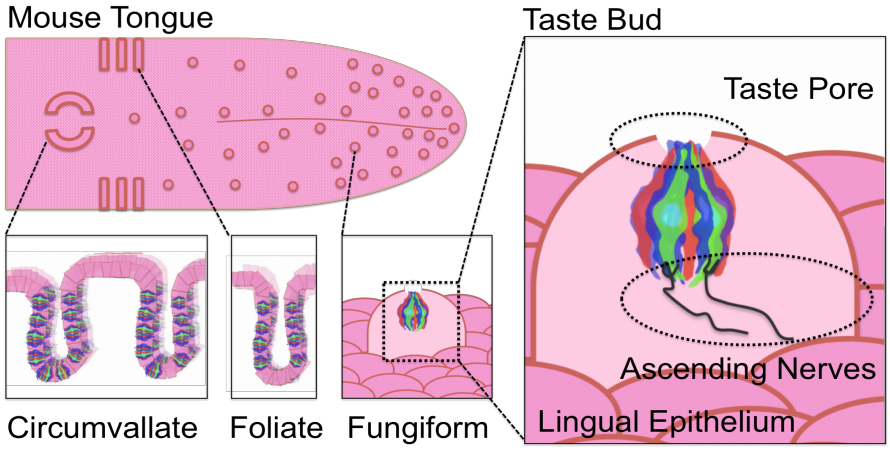

Figure 1. Tongue anatomy and taste bud localization.

Modulators of taste signaling and perception

Obesity status, circulating peptides, and immune response-associated molecules have been shown to alter taste perception. Circulating hormones and cytokines can bind directly to cognate receptors on taste cells to affect taste cell activation and

downstream signaling to afferent nerves. Peptides and their receptors, localized in taste buds, include GLP-1, glucagon, CCK, VIP, NPY, PYY, and ghrelin; though not all have been studied for behavioral effects (reviews: Dando 2010, Dotson et al 2013, Cai et al 2014). The role of pro-inflammatory proteins such as Toll-like receptors (Camandola and Mattson 2017), tumor necrosis factor-α (TNFα; Feng et al 2012), immunological response activators such as interferon receptors (Wang et al 2009), and anti-inflammatory proteins such as interleukin-10 (Feng et al 2014) are beginning to be investigated. Lastly, diet induced obesity decreases CD36 (a putative receptor for the oral detection of fat) expression in taste buds (Zhang XJ et al 2011) and decreases taste responses in mice (Maliphol et al 2013).

Taste affects food preferences, which in turn affect food choice

As previously mentioned, some of the peripheral signaling factors have been implicated in the regulation of taste signaling and perception. The brief schematic from Portella et al (2012) outlines the regulation of food preferences (Figure 2).

Evidence for the maternal diet programming of offspring taste

The developing offspring can be affected by the maternal diet throughout the prenatal period during gestation (in utero) and/or lactation (maternal milk). Pregnant women who consumed carrot juice during the third trimester or during lactation had babies that (when tested at ~6 months of age) preferred and consumed more carrot-flavored cereal when compared to babies who were not maternally exposed to carrots. The no-carrot babies were equally unaccepting of both the carrot and non-carrot cereal (Mennella et al 2001).

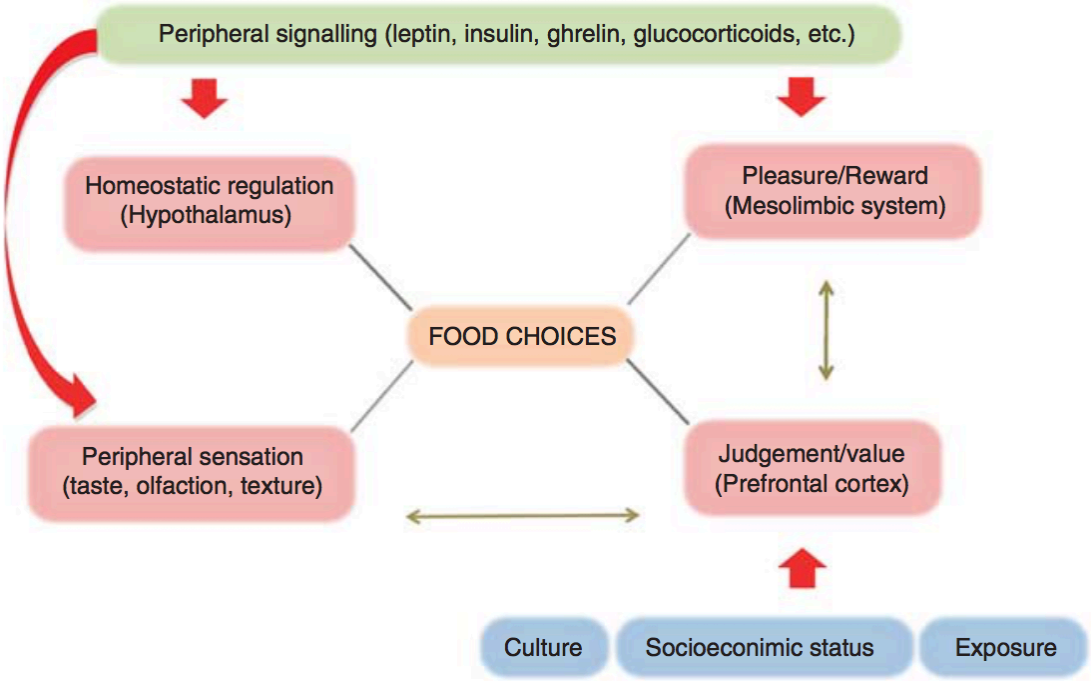

Figure 2. Schematic outlining factors that regulate food preference. The red arrows and red squares, such as the peripheral sensations of taste represent factors that are predominantly centrally mediated. The green highlights regulators that are peripherally mediated, such as adipose tissue, pancreas, gastrointestinal tract, and the hypothalamus–pituitary–adrenal axis.

Rodent studies by Zhang GH et al (2011) demonstrate that acesulfame-K, one of the most widely used non-nutritive sweeteners, is ingested prenatally through the mother's amniotic fluid, as well as postnatally through breast milk, eventually increasing the offspring's preference for acesulfame-K by ~25%, as well as for sucrose by ~30% in two-bottle preference tests. Although follow-up studies focused on early intraoral acesulfame-K exposure in pups instead of *in utero*, they were able to show changes in offspring taste buds for regulators and components of sweet signal transduction such as T1R2, leptin (OB-Rb) and endocannabinoid (CB1) receptors (Li WL et al 2013). Interestingly, an increase in Gα-gustducin expression, believed to be a reliable marker for chemosensitive cells, was found in fungiform taste buds (Chen et al 2013). This

research suggests that maternal ingestive behavior contributes to offspring taste programming.

Maternal high fat diet programs an obesogenic phenotype in the offspring

It is now generally accepted that maternal high fat diet (HFD) produces an unfavorable intrauterine environment associated with the development of metabolic syndrome and an increased risk of obesity in the offspring (Muhlhausler and Ong 2011). We are only beginning to understand the underlying mechanisms that ultimately produce structural and functional changes that in turn drive physiological damage and chronic disease. Considering that maternal HFD during the prenatal period involves both exposure to excess fat in the diet as well as potential weight gain by the mother, it will be difficult to draw conclusions regarding which is the primary driver of this phenotype – diet or adiposity. What is clear is that a number of maternal programming effects have been consistently found and believe to predispose the offspring to develop obesity and metabolic disorder (Figure 3). We focused on the hyperphagic phenotype observed in the adult offspring. In studies of non-human primates (Rivera et al 2015), rats (Treesukosol et al 2014), and mice (Teegarden et al 2009), the mothers given a palatable obesogenic diet resulted in a hyperphagic (i.e. overeating) phenotype for the palatable diet in the offspring. Given these observations, we investigated whether taste may be a contributing factor in maternal obesity induced hyperphagia (Figure 3).

Epigenetic regulation is likely a critical link between maternal diet and the obesogenic phenotype, supporting the hypothesis of the fetal origins of adult disease, also known as Barker's theory, and more recently the "Developmental Origins of Health and Disease (DOHaD)." The DOHaD theory is that environmental factors or an external

stimulus during a critical window of developmental plasticity can generate genotypic variation to change the capacity of the organism to be able to cope better to its environment later in life (Taylor and Poston 2007, Barker 2004, Langley-Evans and McMullen 2010). Evidence suggests that the maternal environment can affect gene transcription partly through epigenetic modulations including DNA methylation, multiple types of histone modifications, and via microRNAs (Lillycrop 2011; Pan et al 2013; Zhang et al 2009). Furthermore, widespread epigenetic programming has been studied in the offspring of models of maternal obesity and diabetes (Li et al 2013).

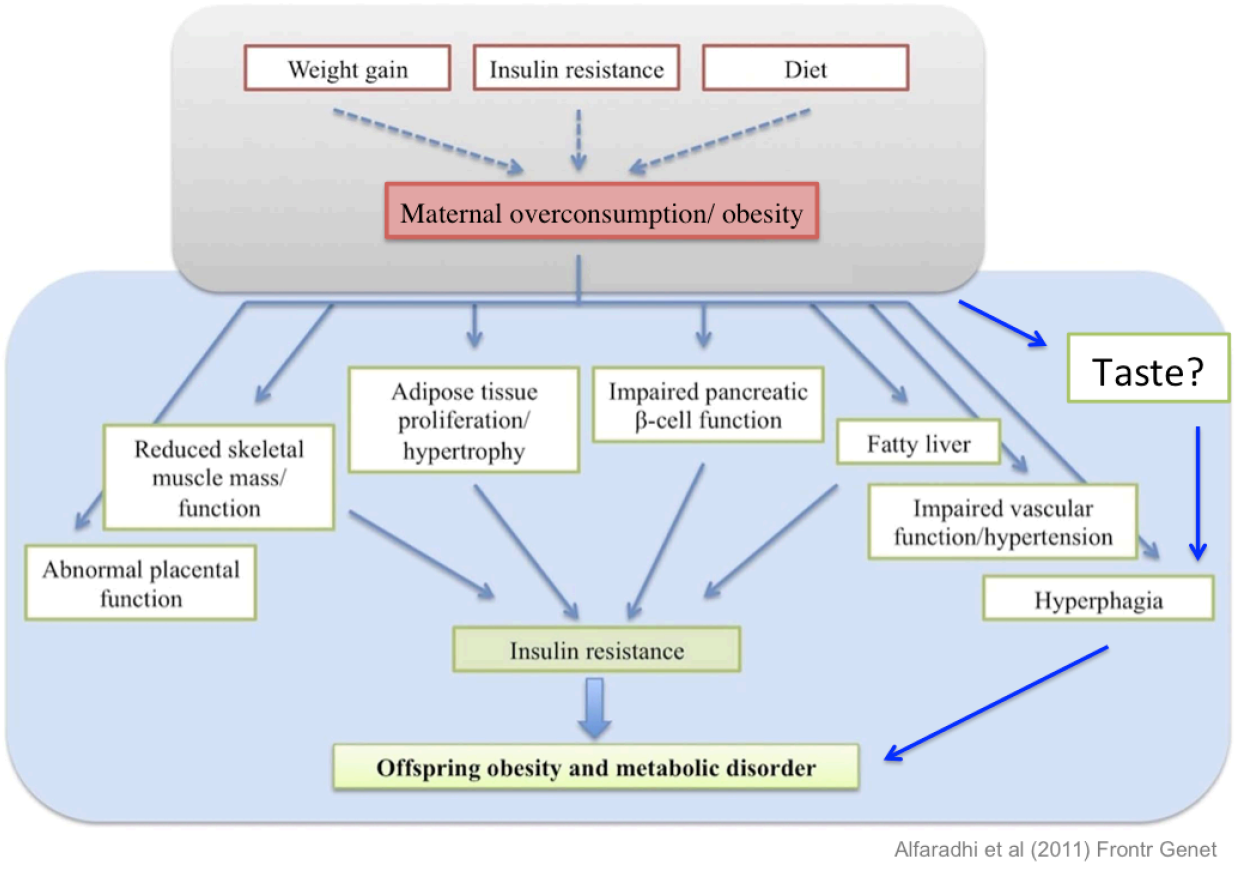

Figure 3. Maternal programming effects that predispose offspring to develop obesity. We investigated whether taste may contribute to the hyperphagic phenotype observed in the offspring as a result of maternal overconsumption and/or obesity.

Sex differences in maternal programming

Any maternal insult, such as that through maternal stress or diet, can directly affect the F1 generation through the placental interface. Depending on the sex of the offspring, the external stimuli can also influence the F2 generation through contact with the primordial germ cells (Figure 4). Thus, the extent of the insult on the epigenome passed on to future generations can also depend on the sex of the offspring (Dunn et al 2011).

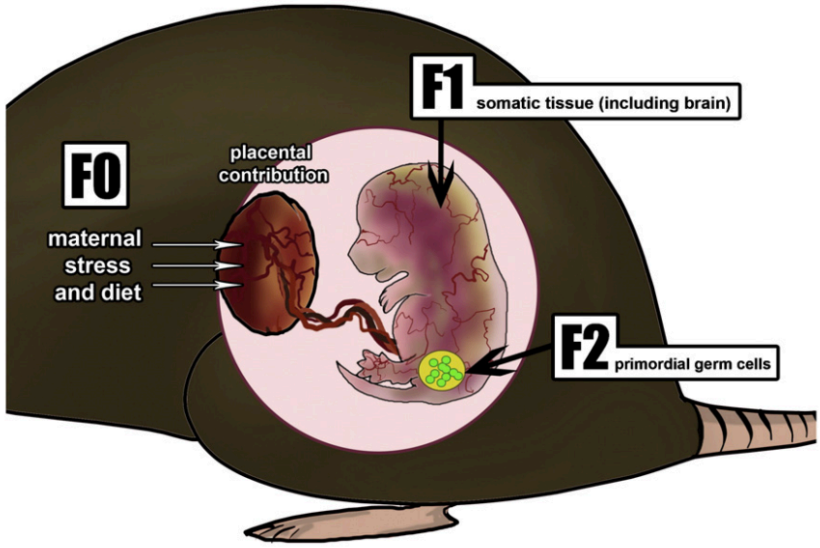

Figure 4. Maternal stress and diet during gestation can be passed to the F1 fetus developing in utero. If the fetus is female, then the maternal environmental stressors could come into direct contact with the F2 through exposure to the primordial germ cells. Adapted from Dunn et al 2011.

Overall Research Objective

The ability to discern appetitive and aversive foods through taste is a process that forms before the offspring is born. Dietary choices, including unhealthy ones, are passed through the amniotic fluid to the developing fetus during pregnancy. Research suggests that gestational diet programs offspring feeding behavior, which persists into adulthood. Given the increasing rates of obesity and the high consumption of fat and

energy-rich foods, it is vital to examine the development of offspring taste perception in the context of maternal obesity and nutrient imbalance during pregnancy.

The goal of this proposal is to determine the impact of pregnancy on maternal taste function, and the long-term effects of maternal fat or sucrose consumption on offspring taste programming. We hypothesize that maternal HFD exposure can be transmitted to the fetus, with effects that persist into adulthood without reinforcement of the stimuli early in life. Our hypothesis would suggest that taste intervention might be a useful strategy to enhance offspring fitness through maternal intake, or paternal health. The results of this proposal will provide a molecular basis supporting significant behavioral research, which works towards an understanding of the development of taste perception and preference. The aims of this project include the following:

Aim 1) characterize maternal taste buds during pregnancy

Aim 2) investigate associations between both maternal HFD and

Aim 3) sucrose consumption on offspring taste perception

This is the first study, to our knowledge, to study these topics at the levels of behavior, morphology, and gene expression. Exposure to excess nutrients before birth predisposes offspring to develop obesity. Adult offspring of obese dams that chronically consume a high-fat diet have increased preference for high-fat diet when compared to their counterparts of lean dams (Rivera et al 2015, Treesukosol et al 2014, Teegarden 2009). A greater understanding of how the maternal diet contributes to long-term changes in the offspring taste system is critical for finding solutions to overcome diseases related to overnutrition and to promote healthy eating habits for children who struggle with obesity and diabetes more now than in previous generations. In spite of

the importance of an increased preference for high-calorie foods in driving the development of obesity, taste behavior and molecular alterations to taste buds of the adult offspring of obese dams have yet to be investigated.

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CHAPTER 2

The Impact of Pregnancy on Taste Function

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Abstract

It is common for women to report a change in taste (for instance an increased bitter or decreased sweet response) during pregnancy, however specifics of any variation in taste with pregnancy remain elusive. Here we review studies of taste in pregnancy, and discuss how physiological changes occurring during pregnancy may influence taste signaling. We aim to consolidate studies of human pregnancy and “taste function” (studies of taste thresholds, discrimination, and intensity perception, rather than hedonic response or self-report), discussing differences in methodology and findings. Generally, the majority of studies report either no change, or an increase in threshold/decrease in perceived taste intensity, particularly in the early stages of pregnancy, suggesting a decrease in overall taste acuity when pregnant. We further discuss several non-human studies of taste and pregnancy that may extend our understanding. Findings demonstrate that taste buds express receptors for many of the same hormones and circulating factors that vary with pregnancy. Circulating gonadal hormones or other contributions from the endocrine system, as well as physiological changes in weight and immune response could all bear some responsibility for modulation of taste during pregnancy. Given our growing understanding of taste, we propose that a change in taste function during pregnancy may not be solely driven by hormonal fluctuations of progesterone and estrogen, as many have suggested.

Introduction

The impact of insufficient or excessive weight gain during gestation on both maternal and offspring health is of great importance (Kaiser et al. 2009). Thus, there exists an imperative need to consolidate research across disciplines to study the role taste plays in food selection throughout pregnancy. Through this review, we will focus on some key studies of taste during pregnancy.

The idea of pregnancy impacting taste is not unusual given that changes in other sensory modalities during pregnancy and labor are widely reported. Women during childbirth report an increase in pain detection thresholds, suggestive of a mechanism to attenuate the pain of parturition (Whipple et al. 1990). The nose may become engorged, causing pregnant women to experience nasal stuffiness and congestion (Bende and Gredmark 1999, Ellegård and Karlsson 1999, Philpott et al. 2004). Taste and smell, while being fundamentally separate systems, work together to shape feeding behavior and dietary intake. It is also believed, at least anecdotally, that pregnant women are hyperosmic; although, there is less evidence to support this (see review by Cameron 2014).

Many women report physiological changes to the mouth during pregnancy. In a questionnaire administered to pregnant women, about half reported concerns about salivary secretion and 63% reported feelings of dry mouth, often linked with taste (Kuga et al. 2002). Assessment of unstimulated whole saliva during pregnancy reveals that various salivary proteins and hormones peak across pregnancy (Muramatsu and Takaesu 1994, Salvolini et al. 1998) and pH and flow rates decrease (Laine et al. 1988, Rockenbach et al. 2006). The degree to which this influences taste function during

pregnancy remains to be determined. Beyond the mouth and saliva, taste can be influenced by genetics, culture, weight, age, hormones, and various aspects of health. During pregnancy, the maternal physiology undergoes a host of adaptations to support fetal development and growth, as well as to ensure that the fetus receives adequate nutrition. These physiological changes include adjustments to the endocrine system, weight gain, increased blood volume, and immune tolerance. Interestingly, many of these factors are also implicated in altering taste perception.

Non-human models of pregnancy provide the opportunity to study taste from behavior to morphology and gene expression. Histological studies examining the lingual papillae of pregnant rats with scanning electron microscopy found topographic changes such as deeper circular sulci around the circumvallate papillae and larger taste pores in the center of fungiform papillae in pregnant rat compared to controls (Yücel et al. 2002). Although not directly tested, researchers suggested that the apparent morphological differences might be due to a variation in hormonal levels. In studies of gestating and lactating nulliparous Long-Evans rats using brief access “lickometer” testing, the pregnant rats had an increased response to salt taste (Clarke and Bernstein 2001). Di Lorenzo and Monroe (1989) looked at electrophysiological responses to sweet, bitter, sour and salty in the parabrachial nucleus of the pons (PbN) of pregnant rats, diestrous female rats, and male rats. The researchers sought to test whether the hormonal state was reflective of changes in processing within the gustatory system. While they did find differences between males and the combined group of females, they found no significant difference between pregnant and non-pregnant rats.

Studies of the fruit fly Drosophila melanogaster found that upon mating, gravid females become attracted to the taste and smell of polyamines such as those from overripe and fermented fruits, which taste sour to mammals (Hussain, Zhang et al. 2016). This increase in polyamine consumption supports reproductive success in the form of increased number of progeny (Hussain, Zhang et al. 2016), providing some support for the idea that taste changes during pregnancy may be beneficial, promoting offspring fitness. This attraction to polyamines may be modulated through a G-protein coupled receptor, the sex peptide receptor (SPR), and its neuropeptide ligands, myoinhibitory peptides (MIPs) acting directly on olfactory and taste neurons that detect polyamines (Hussain, Üçpunar et al. 2016). During human pregnancy, polyamine consumption can be beneficial due to a role in cellular growth, normal cell function, proliferation, and embryonic development (Kalač & Krausová 2005, Lefévre et al. 2011). Fruits, cheese, and fermented foods are relatively high in polyamines. Human studies have shown that high intakes of polyamines in the first year of childhood correlates with food allergy prevention (Dandrifosse et al. 2000). While little work exists on maternal diets high in polyamines, researchers note an elevated level of putrescine (~2x) and spermine (~75x) in the urine of pregnant women, peaking at 12 weeks of gestation (Russell et al. 1978), perhaps justifying either higher intake or synthesis.

The findings from these studies of non-human pregnancy and taste are intriguing and implicate that taste during pregnancy may be subject to modulation from more than just progesterone and estrogen. Thus, we review existing studies of human pregnancy and taste to see what are the trends in taste with pregnancy, and how we may leverage the findings from human and non-human research to generate hypotheses for future

study. Given our growing understanding of taste, we also seek to challenge the assumption that the impact of pregnancy on taste is solely driven by fluctuations in hormones.

Challenges in testing taste during pregnancy

The majority of evidence for a change in taste function during pregnancy arises from self-report, where more than 90% of pregnant women report experiencing some change in taste during pregnancy (Kuga et al 2002). From here on, we will examine what is known about alterations in taste from direct sensory testing of women (not including retrospective surveys or self-reports), with a focus on research of non-pregnant and pregnant women via taste thresholds (detection, recognition) and/ or suprathreshold taste intensity ratings (to both tastant solutions and real foods). Although challenging to carry out, a longitudinal study of taste before, during, and after pregnancy with the same women is subject to the least inherent variability when studying taste in pregnancy. Table I illustrates some of the study designs employed by various groups discussed in this article. A common alternative approach was to compare pregnant women to a separate group of non-pregnant controls. This can represent a weakness in study design, as taste response may be influenced by additional factors such as genetic variation between panelists, or their menstrual cycle (Duffy et al 1998). Although not discussed in depth here, it is also important to consider the stimuli used for testing, see Table II for a comparison of various studies. Differences in testing methodology also offer challenges in comparing results across studies. Finally, it is important to consider the stage of pregnancy as the pregnant body undergoes progressive changes in hormonal balance, weight gain, and immune modulation across the trimesters. An

overview of the population size and stage of pregnancy investigated is also summarized in Table I.

Taste across Pregnancy

To our knowledge, the study of taste across pregnancy by Duffy et al (1998) remains the only longitudinal study analyzing the same cohort of women before pregnancy, and on through each trimester. Duffy et al (1998) tested suprathreshold taste intensity ratings in 46 females before pregnancy and during each trimester, as well as 41 healthy female controls at corresponding time points to assess levels of inherent variation. Interestingly, the control group showed greater variation in sweet and bitter ratings than the pregnant women, which the authors suggest may be associated with the fluctuations in estrogen and progesterone levels across the menstrual cycle. Thus, comparing pregnant women to a different non-pregnant control group, especially one not controlled for timing of the menstrual cycle will likely generate more variation than would internal controls in a longitudinal study design. Other common approaches in the literature include a cross-sectional design comparing non-pregnant women to women in different trimesters and/or postpartum, or a hybrid study design in which the same women are tracked across trimesters/ postpartum with results were compared to a separate group of non-pregnant women. The postpartum period, a time in itself with some hormonal/physiological significance, cannot be assumed to be equivalent to a pre-pregnancy state as it remains unknown whether pregnancy has long-term lasting effects on taste that extend postpartum. Given the increased variation inherent in other study designs, we discuss all other studies in relation to what was found in the longitudinal study by Duffy et al 1998. It is important to note that not all studies looked at

all basic tastes, and that both umami and fat taste in pregnancy warrant further investigation.

Of the basic tastes, sweet taste has been the most studied in regards to pregnancy. Duffy et al (1998) found pregnancy left sweet and sour taste unchanged. Studies by Belzer et al (2010), Brown and Toma (1986), Nanou et al (2016), Ochsenbein-Kölble et al (2005), Saluja et al (2014) and Tepper and Seldner (1999) similarly report no change in sweet taste across trimesters and postpartum. However, several studies report decreased sweet taste in pregnant women (Hansen and Langer 1935 and Landman et al. 1980) with the decrease being specific to the 1st trimester (Kölble et al. 2001 and Kuga et al. 2002). Others found sweet intensity ratings decreased later in pregnancy during the 2nd (Tepper and Seldner 1999) and 3rd trimesters (Saluja et al. 2014), but the results did not reach statistical significance. In contrast to all other studies, Bhatia and Puri (1991) found sweet taste to increase during the 1st trimester in comparison to non-pregnant and pregnant women in their 2nd and 3rd trimesters.

Sour taste was either unaffected by pregnancy (Duffy et al, 1998; Ochsenbein-Kölble et al, 2005; Saluja et al, 2014) or was decreased in pregnant women (Hansen and Langer 1935 and Landman et al. 1980) with any reported decrease being specific to the first trimester (Kölble et al. 2001 and Kuga et al. 2002). The variation in findings may be explained in some part by the differences in methodology, for instance the time at which pregnant women were tested, how they were tested, and whether pregnant women were compared in a longitudinal, or a cross-sectional/case-control design.

Table I. Summary of studies of taste during pregnancy

Authors	Non-pregnant	Pregnant	Design	Stage(s) of Pregnancy studied					
				Pooled	Non-pregnant	1st tri	2nd tri	3rd tri	Post-partum
Duffy et al. (1998)	41	46	**longitudinal**						
Ochsenbein-Kölble et al. (2005)	46	44	**hybrid**						
Kuga et al. (2002)	30	32	**hybrid**						
Belzer et al. (2009)	19	93	**hybrid**						
Tepper and Seldner (1999)	12	30	**hybrid**						
Bhatia and Puri (1991)	Unclear	50 per trimester	**multi-cross-sectional**						
Kölble et al. (2001)	59	53	**cross-sectional**						
Saluja et al. (2014)	30	30	**cross-sectional**						
Landman et al. (1980)	47	104	**cross-sectional**						
Brown and Toma (1986)	23	23	**cross-sectional**						
Nanou et al. (2016)	45	46	**cross-sectional**						

Studies are listed in order of strongest level of evidence, starting with a longitudinal design where the same cohort of women were tested, then hybrid studies that investigated and analyzed different time points of pregnancy but in different groups of women, followed by comparisons of non-pregnant and pregnant women. The greyed boxes in the right-hand side grid represent the stage of pregnancy of the women studied. The grey colors represent different groups of women within an individual study, i.e. did the study assess the same cohort at different time, or a separate cohort at different pregnancy stage. To date, Duffy et al (1998) remains the only longitudinal study to analyze the same group of women before pregnancy and across each trimester. Others (Ochsenbein-Kölble et al. 2005, Kuga et al. 2002, Belzer et al. 2009, and Tepper and Seldner 1999) did collect longitudinal data across pregnancy however were not able to compare their findings with the pre-pregnant state.

Table II. Tastants and concentration ranges of solutions used to test for sweet, salty, bitter, sour, and fat taste reported (M).

	Sweet		Salty		Bitter		Sour		Fat	
Authors	Tastant	Conc.	Tastant	Conc.	Tastant	Conc.	Tastant	Conc.	Tastant	Conc.
Duffy et al. (1998)	Sucrose	0.1 - 1	NaCl	0.1 - 1	QHCl	0.0001 - 0.001	Citr Ac	0.0032 - 0.032		
Kuga et al. (2002)	Sucrose	0 - 2.3	NaCl	0.005 - 0.34	QHCl	0.000003 - 0.01	Tart Ac	0.0001 - 0.053		
Saluja et al. (2014)	Sucrose	0.00001 - 0.001	NaCl	0.00001 - 0.001	QHCl	0.00001 - 0.001	Citr Ac	0.032 - 0.32		
Brown and Toma (1986)	Sucrose	0.01 - 0.25	NaCl	0.025 - 0.2						
Bhatia and Puri (1991)	Glucose	0.063 - 2.0			PTC	0.0000001 - 0.00085				
Tepper and Seldner (1999),	Sucrose	0 - 0.3							Veg. oil	0 - 10 %
Belzer et al. (2009)	Glucose	0.01 - 0.16								

Studies by Landman et al. (1980), Kölble et al. (2001), and Ochsenbein-Kölble et al. (2005) did not report concentrations.

Interestingly, Duffy et al (1998) found salt intensity ratings decreased during the 2nd and 3rd trimester compared with before pregnancy and the 1st trimester. A reduction in the unpleasantness of citric acid during the 2nd and 3rd trimesters was implied to promote an increase in electrolyte ingestion, associating with frequently reported cravings for salty foods such as pickles, which promote fluid expansion (Duffy et al. 1998). In one of the earliest studies of pregnancy and taste, Hansen and Langer (1935) reported that pregnant women have increased thresholds for salt. Brown and Toma (1986) recruited 23 pregnant women and asked them to rank salty solutions from weakest to strongest in “sip and spit” tests, reporting whether women ranked correctly or incorrectly. Eleven of the pregnant women ranked the salt solutions incorrectly, while only 2 non-pregnant women ranked the salt solutions incorrectly. These results, similar to the earliest studies by Hansen and Langer (1935), suggest that salt taste function may be impaired in pregnancy. Interestingly, pregnant women also preferred stronger salt solutions than non-pregnant women (Brown and Toma 1986). In slight contrast, Kölble et al (2001) and Kuga et al (2002) found salt to be decreased during the 1st trimester instead of the 2nd and 3rd as reported by Duffy et al (1998), while Landman at al (1980), Ochsenbein-Kölble et al (2005), Saluja et al (2014) found no impact of pregnancy on salty taste.

The taste modality with the least consensus in pregnanmcy is bitter. Across trimesters, Duffy et al (1998) and Bhatia and Puri (1991) both found the perceived intensity of bitter to be increased during the 1st trimester. Duffy et al (1998) found bitter intensity ratings rose in the 1st trimester and then fell in the 2nd and 3rd. The authors postulated that the elevation in bitter intensity in the 1st trimester might help pregnant

women avoid toxins during the critical phase of early fetal development. This increase in bitter taste sensitivity during may protect the mother and fetus from foodborne illnesses or the consumption of toxins (Profet 1992, Flaxman and Sherman 2000). Several commonly reported food aversions during pregnancy are high in teratogenic or abortive potential (Profet 1992, Fessler 2002), thus the protective association of nausea and vomiting in early pregnancy may carry a reduced risk for pregnancy loss (Hinkle et al 2016). While the above studies found an increased bitter response in the 1st trimester, both Hansen and Langer (1935) and Landman et al (1980) report bitter response decreased across pregnancy, whereas Ochsenbein-Kölble et al (2005) found hypogeusia for bitter during the 1st trimester, that persisted through the postpartum period. This study follows-up on earlier work by Kölble et al (2001) that used similar testing methods, and finding bitter taste decreased in the 1st trimester. In addition to testing more time points, Ochsenbein-Kölble et al (2005) tested the non-pregnant controls during the second half of the menstrual cycle in order to avoid the early luteal phase when estrogen levels are relatively high; a period which may influence taste perception and has previously been characterized by increased cravings for sweets (Doty 1978, Prutkin et al. 2000). In contrast to findings stating that bitter varies across pregnancy, Nanou et al (2016) and Saluja et al 2014 instead report that pregnancy did not impact bitter taste. Interestingly, Kuga et al (2002) measured gustatory thresholds of regions innervated by the chorda tympani (a branch of VII) and glossopharyngeal (IX) nerve in 32 pregnant (tested serially over each trimester period) and 30 non-pregnant women and found bitter, sweet, salty and sour to be decreased in the 1st trimester in the glossopharyngeal region (the tonsils, pharynx, and the posterior one-third of the

tongue), but no change to bitter or salty for the region innervated by the chorda tympani. Finally, Kuga et al (2002) detail a case report of longitudinal electrogustometric testing of a single subject from the 9th to the 33rd week of pregnancy. Both gustometer and tastant thresholds were elevated early in pregnancy, interestingly with corresponding report of a preference for strongly flavored foods.

Overall, findings are intriguing, however a clear consensus is lacking. The only longitudinal study to date found bitter increased in the 1st trimester and salt decreased in 2nd and 3rd trimester (Duffy et al 1998); however, only one other study reports an increase in bitter taste (Bhatia and Puri 1991) while other studies do not confirm a decrease in salt taste. This is in opposition to the trends suggested by other authors (see Table III) of no change or a weakening of taste function during pregnancy, particularly during the 1st trimester. While all authors sought to evaluate taste during pregnancy, a likely reason for this disagreement in findings is that individual variation was not controlled for aside from in the longitudinal design by Duffy et al 1998. The degree to which taste function must shift to cause a change in food preference or feeding behavior also remains unclear.

Table III

	Decreased Function					No Change					Increased Function
	Mixed Pregnancy Stage	**1st Trimester**	**2nd Trimester**	**3rd Trimester**	**Postpartum**	**Mixed Pregnancy Stage**	**1st Trimester**	**2nd Trimester**	**3rd Trimester**	**Postpartum**	**1st Trimester**
SWEET	Hansen Landman	Köble Kuga (ct) Kuga (gl)				Brown Nanou	Duffy Ochsen-K	Belzer Bhatia Duffy Kuga (ct) Kuga (gl) Ochsen-K	Belzer Bhatia Duffy Kuga (ct) Kuga (gl) Ochsen-K Saluja Tepper	Belzer Ochsen-K Tepper	Bhatia
BITTER	Hansen Landman	Köble Kuga (gl) Ochsen-K	Ochsen-K	Ochsen-K	Ochsen-K	Nanou	Kuga (ct)	Duffy Bhatia Kuga (ct) Kuga (gl)	Duffy Bhatia Kuga (ct) Kuga (gl) Saluja		Duffy Bhatia
SOUR	Hansen Landman	Köble Kuga (ct) Kuga (gl)					Duffy Ochsen-K	Duffy Ochsen-K Kuga (ct) Kuga (gl)	Duffy Ochsen-K Kuga (ct) Kuga (gl) Saluja	Ochsen-K	
SALT	Brown Hansen	Köble Kuga (gl)	Duffy	Duffy		Landman	Duffy Kuga (ct) Ochsen-K	Kuga (ct) Kuga (gl) Ochsen-K	Kuga (ct) Kuga (gl) Ochsen-K Saluja	Ochsen-K	

ct = chorda tympani, gl = glossopharyngeal. 1st Trimester (<15 weeks), 2nd Trimester (16-27 weeks), 3rd Trimester (28 week-birth), Postpartum (varied between studies from 6-12 weeks post partum). Abbreviations of author names from Table I are used here. Studies include taste threshold testing or scaling with pregnant women comparing before and after pregnancy, or comparing pregnant to a separate non-pregnant control group of female panelists. Note, not all studies investigated the same tastants or concentrations, see Table II.

Endocrine Factors and Their Effect on the Peripheral Taste System

The discovery over the last few decades of endocrine receptors in taste buds has changed the way we think about taste. Some have hypothesized that the ovarian hormones estrogen and progesterone may play a role in craving etiology during pregnancy (Orloff and Hormes 2014), however, a direct role is yet to be proven. The following section summarizes direct modulators of taste function that increase in pregnancy, many of which have been studied using non-human animal models (Table IV). For an extended general discussion of the endocrinology of taste, consult reviews by Calvo and Egan (2015), Dando (2010),and Loper et al. (2015).

The oxytocin receptor has been described in taste buds, with several recent studies suggesting that oxytocin influences sweet taste response. The oxytocin receptor (OXTR) is expressed in type I taste cells, with oxytocin likely delivered through the circulation, rather than being produced locally in the taste bud (Sinclair et al. 2010). Studies of oxytocin knockout (KO) mice reveal that without oxytocin regulation, KO mice will consume significantly larger amounts of both sweet and non-sweet carbohydrate solutions than their wild type counterpart (Sclafani et al. 2007). Further studies by Sinclair et al (2015) suggest that oxytocin acts on OXTRs in taste to dampen peripheral sweet taste responses. Although oxytocin is commonly known for its role during labor to stimulate the powerful contractions necessary for the birthing process, levels of oxytocin gradually increase across each trimester.

Leptin is known as a satiety hormone, produced primarily by white adipose cells to inhibit feeding. Studies by Kawai et al. (2000) show that the leptin receptor (Ob-R) is expressed in type II taste cells. Leptin administration in lean mice suppresses peripheral

taste nerve responses from the chorda tympani and glossopharyngeal nerve to sweet substances including non-nutritive saccharin, without affecting responses to other tastants, suggesting that leptin can selectively decrease sweet taste sensitivity (Kawai et al. 2000). Leptin levels steadily increase during the 1st and 2nd trimesters with increasing adiposity, which may in itself result in changes to taste function (Dando, 2015), peaking in the late 2nd or early 3rd trimester (Hardie et al. 1997, Schubring et al. 1998), while also being synthesized in the placenta. Thus, increasing levels of leptin during pregnancy may act on taste cells to dampen sweet taste sensitivity.

In pregnant women, the renin-angiotensin system plays an important role in regulating blood pressure, electrolyte balance, and the subsequent wellbeing of mother and fetus. Angiotensin II (AngII) is classically known for its role in the regulation of vascular tone, and sodium reabsorption. AngII acts on two receptors, AT1 and AT2, widely distributed in the body. To form AngII, the liver produces angiotensinogen while the kidneys produce renin in response to renal sympathetic activity. Renin cleaves angiotensinogen to create angiotensin I. Subsequently, angiotensin I is converted to AngII by the enzyme angiotensin-converting enzyme (ACE), primarily found within the lungs. During normal pregnancy, all components in the renin-angiotensin system are greatly increased over non-pregnant, except for ACE (Irani and Xia 2008). In taste cells, the AT1 receptor is expressed with some type I taste cells positive for αENaC, as well as some type II taste cells positive for T1R3 (a shared subunit for sweet and umami taste signaling) and TRPM5, suggesting that the taste system may be a peripheral target of AngII (Shigemura et al. 2013). In gustatory nerve recordings, AngII was found to enhance responses to sweeteners, and suppress amiloride-sensitive salt taste

responses, while the other basic tastes (sour, bitter, umami) were unaffected (Shigemura et al. 2013). Given the complex system necessary to produce AngII, it is unlikely that the taste cells can produce AngII, although the production of precursors or ACE is conceivable. Taken together, these findings suggest that the increased levels of AngII during pregnancy may act on taste to increase the intensity of sweet taste perception, and diminish that for salt.

Table IV. Summary of sweet taste modulators linked to pregnancy.

Modulator (*indicates that it is produced in taste cells)	Level during human pregnancy	Source(s)	Receptor present in taste	Localization of receptor	Reference(s)
Oxytocin	increased	Posterior pituitary.	OXTR	Subpopulation type I cells	Sinclair et al. (2010) Sinclair et al. (2015)
Leptin	increased	Primarily adipocytes in white adipose tissue. It is also produced by the placenta.	Ob-R	Type II cells	Kawai et al. (2000) Shigemura et al. (2004)
Angiotensin II	increased	Renin-angiotensin system.	AT1	Type I and II cells	Shigemura et al. (2013)
Tumor necrosis factor-alpha*	decreased	Primarily macrophages, although it is produced by many different cell types, including T1R3+ taste cells.	TNFR1 TNFR2	Most taste cells type I-III	Feng et al. (2012) Feng et al. (2015)
Interleukin 10*	increased	Various cell types. In taste, IL-10 is found exclusively in G-alpha gustducin bitter-sensitive cells.	IL-10R1	IL-10R1 has been reported to be primarily on T1R3+ cells	Feng et al. (2014)

An Altered Immune Response may Influence Taste Function

The correct balance of the immunologic system, through proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and counter regulatory cytokines such as interleukin-10 (IL-10) is essential for the maintenance and development of a normal pregnancy. For a review of the role and actions of cytokines in pregnancy, see Moreli et al (2012). Studies of healthy pregnancy have found a global reduction of proinflammatory cytokines, such as TNF-α, while counter regulatory cytokines, such as IL-10 increase across pregnancy (Denney et al. 2011). Interestingly, it has also been shown that taste buds utilize many parts of the machinery involved in immune and inflammatory signaling pathways. Mouse studies by Feng et al (2012) showed that TNF-α is localized in type II taste cells that co-express the T1R3 subunit for sweet and umami taste signaling, and that localized taste cell-specific production of TNF-α can be modulated by inflammatory activators such as bacterial lipopolysaccharides. The same group carried out follow-up studies using behavioral taste testing and gustatory nerve recordings of TNF-α knockout mice, finding that the immune system regulates sensitivity to bitter taste (Feng et al. 2015). Though their taste bud morphology was comparable to wild type animals, TNF-α deficient mice were found to be less sensitive to quinine in behavioral taste testing, with the taste cells of TNF deficient mice less responsive to both quinine and denatonium, with sweet, umami, salty, and sour unaffected (Feng et al. 2015). A general reduction in TNF-α levels during pregnancy (Denney et al. 2011) or even increases that relate to increased risks for obstetric complications (Moreli et al. 2012) may therefore influence bitter taste. The anti-inflammatory cytokine IL-10 appears not to be expressed in the same taste cells positive for TNF-α and T1R3, but is

instead expressed in type II cells positive for G-alpha gustducin (Feng et al. 2014). Findings by Feng and colleagues suggest that IL-10 may play a critical role in maintaining the structural integrity of the mammalian taste system, as mice deficient in IL-10 had significantly smaller taste buds, and the number of taste receptor cells per taste bud was also reduced. What roles these adaptations in immune function may have on taste during pregnancy are yet to be determined.

Conclusion

In this review, we examined taste in pregnancy from animal models and direct studies of humans, as well as reviewing some of the physiological changes that can influence taste. One conclusion that is plain from our review is that there still remains a need to fully characterize the variation in gustatory function that occurs throughout pregnancy. A deal of disagreement is still evident, possibly due to a lack of agreement on experimental design. The best design remains to test the same cohort of women before, during, and after pregnancy. Integrating studies from animal models with existing theory on the mechanism underlying change to taste with pregnancy may help in advancing our understanding of feeding behavior during this important period. Since many studies found some change in taste during the 1st trimester, usually a small decrease in function, one might be led to assume that this may be due to the sudden increase in hormones at the beginning of pregnancy. We propose that researchers consider other paradigms to explain modulation of the taste system in pregnancy. Additionally, given the expanding repertoire of taste modulators such as hormones, circulating factors, and the immune system, we challenge the assumption that taste is

affected during pregnancy only due to changes in progesterone and estrogen. A better understanding of taste modulation in health and disease may help us better understand the cause and effect of gestational obesity, hypertension, diabetes, and hyperemesis gravidarum (Belzer et al 2009; Tepper and Selner 1999; Sipiora et al. 2000). Further study may enable us gauge more of the consequences of a change in taste function during pregnancy, for example whether taste varies to assist in supporting a healthy pregnancy, and if a change in taste may result in negative consequences to the health of the mother and offspring.

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CHAPTER 3

Role of taste buds in the development of taste sensitivity during mouse pregnancy

Abstract

An alteration in maternal intake during pregnancy permanently affects the metabolism, growth, and feeding behavior of the progeny, in both mice and humans. While much is known about how maternal diet affects offspring fitness, less is known about how gustation is involved in guiding and promoting food intake during this crucial period. Women have intense food cravings and exhibit altered taste preferences during pregnancy. However, the mechanistic details underlying these changes during pregnancy are presently unclear. We performed longitudinal brief-access taste testing in female mice before, during, and after pregnancy and found decreased sucrose licking responses during pregnancy compared to prior to mating ($F_{4,258}$=3.216, p=0.0134) and postpartum ($F_{4,244}$=6.618, p<0.0001). We hypothesize that altered taste preferences during pregnancy results from changes in the expression profile of the taste buds of the mother, and from circulating hormones acting on cognate receptors in taste. We performed qPCR to study taste receptor expression, a potential pathway for the modulation of taste signaling. The results indicate that the decrease in sucrose response may be due to a decrease in taste receptor expression, or in the absolute number of taste buds present in the circumvallate papillae. The signaling of sweet taste is comprised of a network, not a single pathway, so further investigation into other transcripts involved in sweet signal transduction is warranted.

Introduction

The gustatory system can be utilized as a target for regulating food intake and eating behavior. There is a dire need for interventions to motivate women to adopt healthy eating habits during pregnancy (Trout and Effinger 2012), especially given the increasing evidence that those habits may have persistent lifelong effects in their offspring. However, much of our knowledge of taste modulation during human pregnancy is inconsistent. Some studies have focused particularly on sweet taste, suggesting an increase in preference during pregnancy (Worthington-Roberts et al 1989; Pope et al 1992; Bayley et al 2002). Others have implicated a general decline in gustatory function during early to mid pregnancy (Duffy et al 1998; Kölble et al 2001; Kuga et al 2002; Ochsenbein-Kölble et al 2005). Although studies have been performed into the mechanisms involved in the etiology of food cravings in other settings (i.e. pica, menstruation, hypertension, etc.; reviewed by Orloff and Hormes 2014), the role of the taste buds themselves in food cravings during pregnancy remains relatively uncharted. Understanding how taste influences the maternal diet during pregnancy is critical as these experiences set the stage for later food choices and establishing life-long food habits in the offspring.

A host of physiological changes occur during pregnancy that could contribute to changes in taste. Weight status, circulating peptides, and immune-response-associated molecules have been shown to modulate taste perception (Figure 1) and extend the hypothesis that taste changes during pregnancy due to rising hormones such as estrogen and progesterone (Choo and Dando 2017). Interestingly, circulating hormones and cytokines can bind directly to cognate receptors on taste cells to affect taste cell

activation and the downstream signaling to afferent nerves. For example, leptin from adipose tissue (Shigemura et al 2004), circulating oxytocin (Sinclair et al 2010, 2015), and tumor necrosis factor-α (Feng et al 2012), have all been suggested to act on cognate receptors at the taste bud level to decrease sweet responses. As more therapies, through hormonal or inflammatory intervention, are proposed to prevent complications and miscarriage during pregnancy, it will become increasing important to understand how these actions may additionally influence taste and subsequently feeding behavior.

The purpose of this study was to determine how the physiological state of gestation and lactation influenced the short-term sensitivity to sucrose and to investigate any changes to taste bud morphology. Furthermore, we studied the taste transcriptome using qPCR on taste buds collected from pregnant and non-pregnant females to begin probing the complex pathways that drive the taste changes we see during pregnancy.

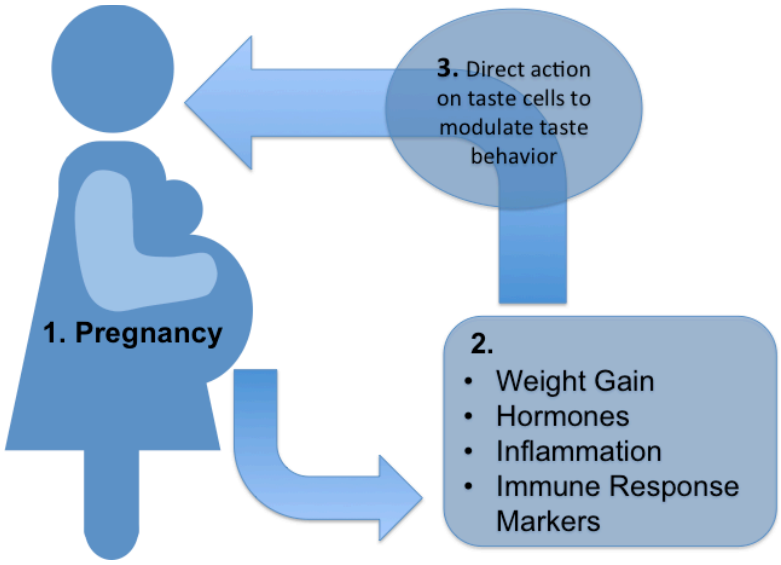

Figure 1. Weight status, circulating peptides, and immune-response-associated molecules have been shown to modulate taste perception and may contribute to changes in taste during pregnancy.

Methods

Animals

In-house bred virgin C57BL/6 females were used in all experiments. At 7 weeks, mice were single housed and given a week to acclimate. Mice were provided *ad libitum* normal chow (18% kcal from fat, Teklad Diets). For mating, females were placed in the male cages. Day 1 of pregnancy was determined by detection of copulatory plug and females were then returned to their home cages. All animal work was approved by the Institutional Animal Care and Use Committee at Cornell University.

Sweet Taste Behavior Assay – Brief-Access Lickometer

Taste responses (i.e. taste-related affective potency of the stimuli) were measured using a brief-access Davis Lickometer, which minimizes confounding factors such as appetite and post-ingestive effects. This method quantifies immediate lick responses to extremely small volumes of sapid solutions and the training and testing schedule was adapted from Glendinning et al 2002 and Glendinning et al 2005. Training and testing were conducted under simulated dark cycle conditions using red lights. To acclimate mice to the Lickometer testing chamber, mice were partially water restricted by providing 1 mL of water for 23.5 hour and then placed in the Lickometer and trained to lick from an available spout containing water for 30 minutes. For two additional days the mice were again partially water restricted and placed in the Lickometer for 45 minutes each day now with the full Lickometer functioning (again only water was in the bottles). Before testing, mice were partially water and food restricted by providing 1 mL of water and 1 g of normal chow for 12 hours prior to tastant training. Partial food and water restriction times were lowered to 12 hours during testing as

recommended by the Institutional Animal Care and Use Committee at Cornell University.

For sweet testing, a range of sucrose (0, 0.03, 0.1, 0.2, 0.3, 0.6, 1.0 M) concentrations was used. The presentations were randomized in blocks so that every concentration was presented once before being repeated again. Once the mouse initiates licking on the presented bottle, the timer started for 5 seconds and then the shutter closed. Each test session lasted no more than one hour, during which the mouse could initiate up to 5 blocks of 7 concentrations (i.e. 35 total presentations). To measure sweet taste responses across pregnancy, mice were tested with sucrose twice before mating, three times (once each week) during gestation, and finally twice during the second and third week of lactation just prior to weaning (Figure 2).

Data were downloaded as .csv files and imported into Excel for further analysis. Lick responses were normalized, fit to nonlinear variable slope concentration-response curves, and compared using extra sum-of-squares F test. The number of licks for each concentration was averaged within each mouse. These averages were then divided by the maximal lick rate and subtracted from the minimum rate within each mouse yielding the standardized lick ratio. A lick ratio of 0.0 indicates that the sucrose concentration elicited minimal licking over water, whereas a value of 1.0 indicates maximal licking; thus, controlling for individual differences in local lick rate for each mouse. Tastant concentration–lick ratio response curves were fitted to the mean data for each group using a classical four parameter logistic sigmoidal dose–response equation in the nonlinear regression suite of GraphPad Prism (v5.0). To examine if there were any

differences in the maximum and minimum responses, all four parameters remained unconstrained (i.e. bottom was not constrained to 0 and top was not constrained to 1).

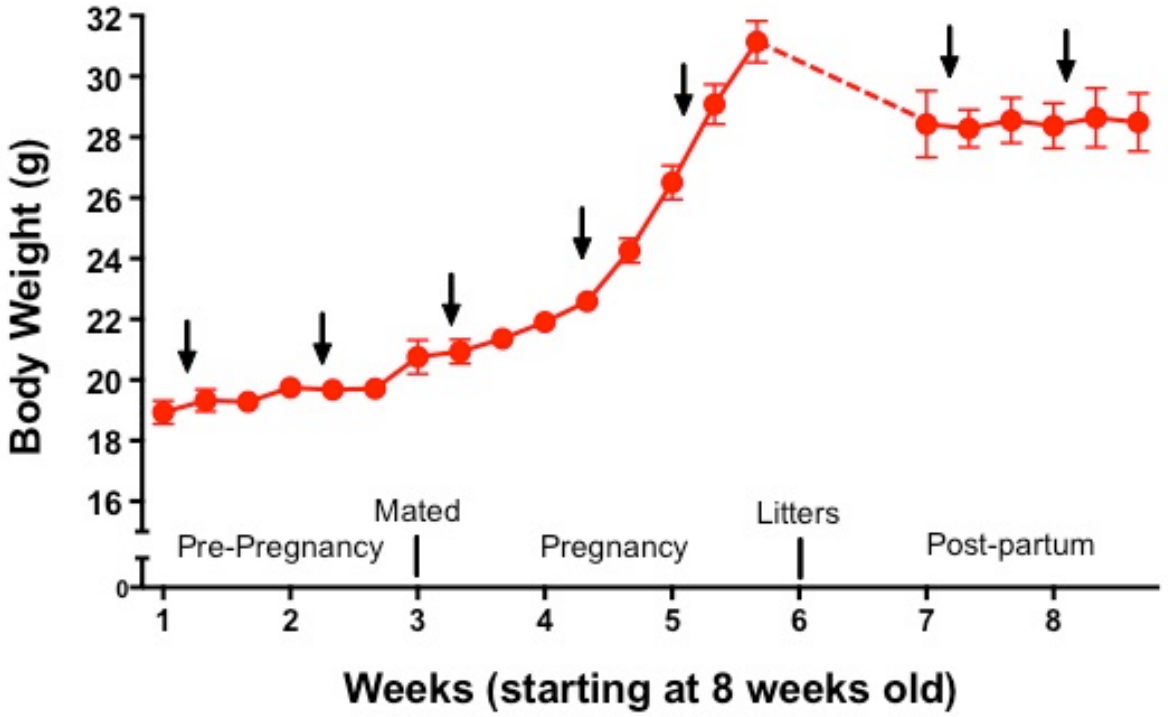

Figure 2. Schematic of Lickometer testing before, during, and after pregnancy. Downward arrows indicate estimate time in which females were tested with sucrose solutions using brief-access Lickometer. Responses were averaged within each mouse for each time period.

RNA extraction, reverse transcription, and determination of gene expression

Total RNA was extracted using Absolutely RNA Nanoprep Kits for taste samples and RNA Microprep Kits for non-taste samples (Agilent, Stratagene) and used as template for cDNA synthesis with qScript cDNA SuperMix (Quanta Bio, Beverly, MA). Quantitative real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) was run on a QuantStudio 6 Flex Real-Time PCR System (Thermo). PLCβ2 enrichment over non-taste samples was used as a positive control for taste cells. Relative quantification was performed in triplicates using QuantStudio PCR

Software, based on the $2^{-\Delta\Delta Ct}$ method. Beta-Actin was used as the endogenous housekeeping gene for normalization of genes of interested (Table 1). To control for false positives, a non-template control was run for each template and primer pair. The treatment groups were compared using t-test, with $p<0.05$ as significant.

Protein	Gene	Forward 5'-3'	Reverse 5'-3'	Product size (bp)
β-actin	*Actb*	caccctgtgctgctcacc	gcacgatttccctctcag	328
PLCβ2	*Plcb2*	gagcaaatcgccaagatgat	ccttgtctgtggtgaccttg	163
T1R1	*Tas1r1*	ctggaatggacctgaatggac	agcagcagtggtgggaac	185
T1R2	*Tas1r2*	aagcatcgcctcctactcc	ggctggcaactcttagaacac	114
T1R3	*Tas1r3*	gaagcatccagatgacttca	gggaacagaaggacactgag	283
TNFα	*Tnfa*	acgtggaactggcagaagag	gaggccatttgggaacttct	179
T2R5	*Tas2r105*	gaatcatagaaacaggacctcg	ctttacaaaggcttgctttagc	406
T2R8	*Tas2r108*	ttctgatttcagccctcacc	ccaaaagctggtcctgtttc	245
α-ENaC	*Scnn1a*	ggcagcccaccgaggagga	gccacagcaccgcccagaa	159

Table 1. Genes of interest and their corresponding primer sequences.

Immunofluorescence and morphometric analyses

The 4% PFA fixed tissue was sectioned at 10 microns and stained with hematoxylin and eosin (H&E) or antibodies for immunofluorescence (Table 2). Images were taken using an Olympus IX-71 microscope with a Hamamatsu Orca Flash 4.0 camera. For immunofluorescence, in brief, tissue was incubated at room temperature with blocking solution (2% BSA, 2% donkey serum, 0.3% Triton) for at least 3 hours, and then incubated at 4°C overnight with primary antibody. AlexaFluor 488, 594, 647-conjugated secondary antibodies raised in donkey against rabbit or goat (1:1000 dilution) for 2 hours of secondary antibody incubations. In order to obtain an unbiased count from taste buds in the circumvallate, every 5th section was used for quantification. The percentage of cells of interest was determined by cell counting. In

brief, random taste buds from the left and right sides of the circumvallate were chosen, with a minimum of 16 taste buds per mouse (n=2 non-pregnant and n=2 gestation day 18). Slides were mounted using DAPI Fluoromount-G (SouthernBiotech). Sox2 high level positive stain was counted by opening the images in Photoshop and then adjusting the midtone input level from 1.00 to 0.3 to enhance the high Sox2 stain and dim the low Sox2 signal.

Antigen	Host	Vender	Dilution
NTPDase2	rabbit	J. Sévigny at Université Laval, Quebec	1:1000
T1R3	goat	Santa Cruz Biotechnology	1:1000
KCNQ1	goat	Santa Cruz Biotechnology	1:1000
Ki67	rabbit	Thermo	1:1000
Sox2	goat	Santa Cruz Biotechnology	1:1000

Table 2. Primary antibodies used for immunofluorescence analysis

Results

Sucrose lick response prior pregnancy, during, and after

Brief-access sucrose lick responses were evaluated in the female mice prior to mating (non-pregnant), after mating during gestation (pregnant), and after litters were born (postpartum). Four parameter nonlinear regression analysis of the concentration-response curves for sucrose showed that the sucrose responses during the pregnant condition were significantly different from when non-pregnant ($F_{4,258}$=3.216, p=0.0134, Figure 3B) and after in postpartum ($F_{4,244}$=6.618, p<0.0001, Figure 3C). Analysis of the data revealed that postpartum was not significantly different from the non-pregnant condition ($F_{4,244}$=1.711, p=0.1480, Figure 3A). The maximum was lower during

pregnancy (0.8820 ± 0.2154) than the pre-pregnant period (1.181 ± 0.2307), while the minimum was similar between pregnancy (0.08173 ± 0.02154) and pre-pregnancy (0.04750 ± 0.04023). The Hill Slope was steeper during pregnancy (2.859 ± 0.5733) than the pre-pregnant period (1.316 ± 0.3787). The relative EC50 prior to pregnancy was 0.3591 ± 0.1566 and during pregnancy was 0.2978 ± 0.0319 (Figure 3B).

Pregnancy responses were overall lower relative to the postpartum period (Figure 3C). The maximum was lower during pregnancy (0.8820 ± 0.2154) than postpartum (1.007 ± 0.04725). The minimum lick ratio during pregnancy was 0.08173 ± 0.02154 and postpartum 0.1169 ± 0.02369. The Hill Slope was similar between pregnant (2.859 ± 0.5733) and postpartum (2.645 ± 0.4991). The relative EC50 prior to pregnancy was 0.3591 ± 0.1566 and during postpartum was 0.2767 ± 0.03102. Because the data was normalized, but the curves not constrained to a minimum value of zero and maximum of one, absolute EC50 values were not determined.

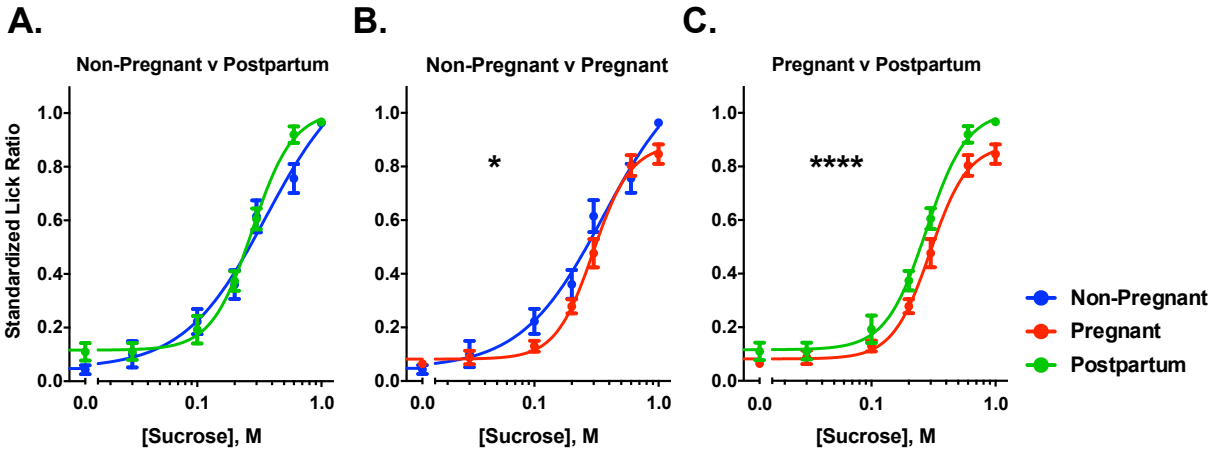

Figure 3. Female mice were tested for brief-access sucrose responses prior to mating, during pregnancy, and after litters were born. Non-pregnant and pregnant response were measured in n=19 mice, while n=17 for postpartum because two females died during the birthing process. Sweet taste responses during the pregnant period were significantly different from non-pregnant and postpartum periods as determined by four parameter nonlinear regression analysis; */**** when $p < 0.05/0.0001$.

Changes in taste bud gene expression

We hypothesized that the behavioral changes we see during pregnancy are due to modulation of gene expression in the taste buds. Relative qPCR was performed on taste bud samples collected from females that had never been mated with males, female mice during gestation day 6, day 12, day 18, and postpartum (n=4-5 for each time point, Figure 4). PLCβ expression was used as a positive control for taste cells. One-way ANOVA for each gene of interest was performed with post-hoc Tukey where $p<0.05$. Analysis indicated gene expression differences amongst the samples for PLCβ ($p=0.0013$), T1R1 ($p=0.0234$), T1R2 ($p=0.0001$), T1R3 ($p=0.0001$), T2R5 ($p<0.0001$), T2R8 ($p=0.0004$), and ENaC ($p<0.0001$). Expression of TNFα was not different among the time points examined (Figure 4B). Because we were interested in how gene expression varied across pregnancy and postpartum periods, post-hoc Tukey significance are shown when comparison was against the group of Not-pregnant females. Specifically, T1R1 was increased about 2-fold during Gt 12 when compared to the not-pregnant group ($p<0.0001$), T1R3 expression was decreased during postpartum ($p<0.001$), T2R6 expression increased in early pregnancy Gt 6 ($p<0.001$) and then decreased at Gt 12 ($p<0.05$), and T2R8 was lower during Gt 12 ($p<0.05$) and postpartum ($p<0.01$, Figure 4B). ENaC was lower during Gt 12 ($p<0.001$) and postpartum ($p<0.01$, Figure 4C). Multiple comparison results of the other time points examined are summarized in Supplement Table 1.

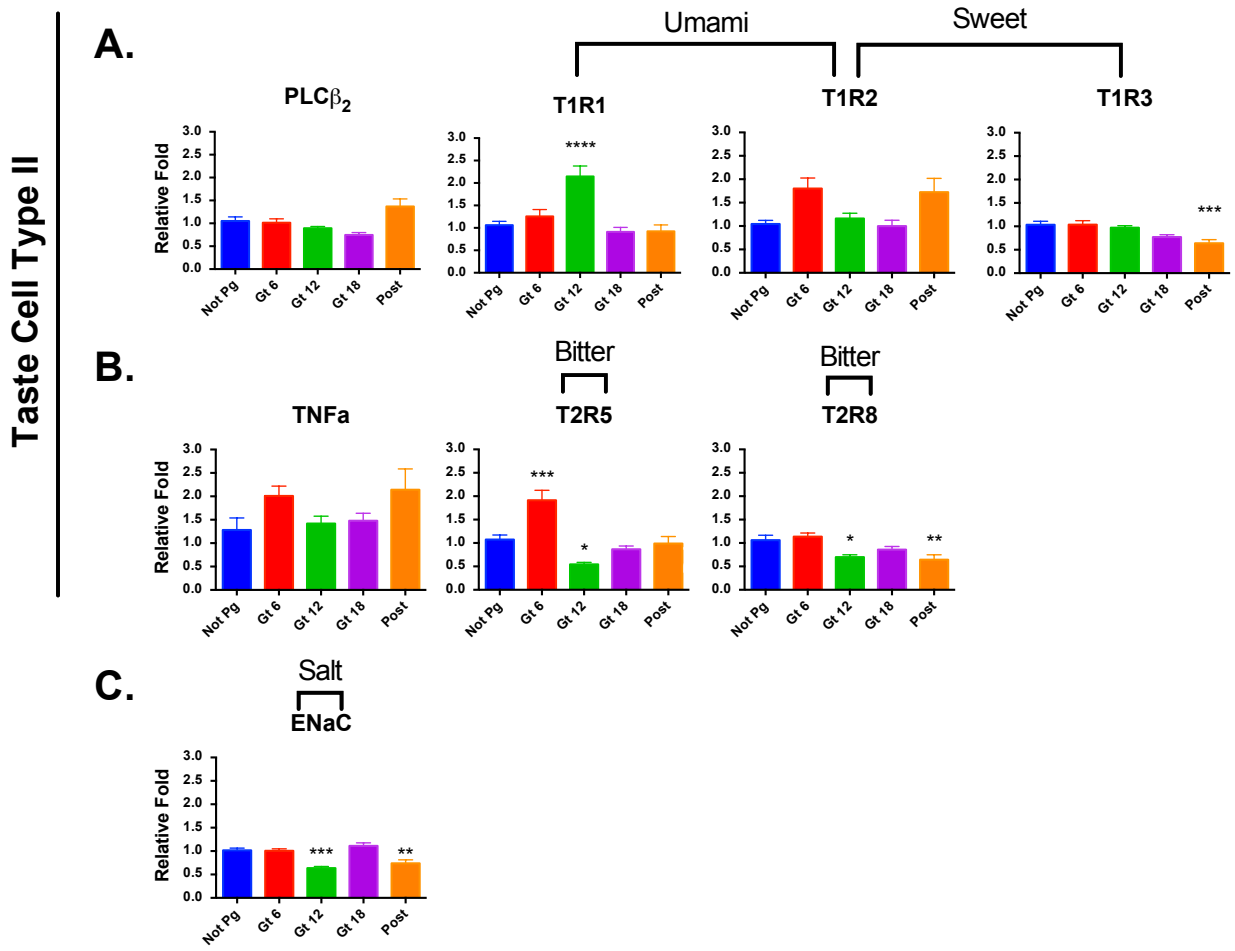

Figure 4. Taste buds were isolated from female mice that had never been mated with males, female mice during gestation day 6, day 12, day 18, and postpartum (n=4-5 for each time point). Data were analyzed with one-way ANOVA and post-hoc Tukey analysis (when ANOVA $p<0.05$). Significant findings compared to the Not-Pregnant group of females are labeled here as */**/***/**** when $p < 0.05/0.01/0.001/0.0001$.

Effect of pregnancy on taste bud morphology

To investigate how pregnancy affects tongue morphology, we first determined whether the treatment cause any alterations to the fungiform papillae density on the surface of the tongue anterior. Increased fungiform density has been correlated with increased taste sensitivity at least in humans (Miller and Reedy 1990). We counted

fungiform density on the anterior tip of the tongues of mice from each treatment group as a measure of taste sensitivity and found no difference in fungiform density between the treatment groups (Figure 5). One-way ANOVA reveals no significant difference between treatment groups for fungiform density (p=0.2336).

Images of H&E staining of tongue tissues were used to measure taste bud size and epithelial thickness (Figure 6C). T-tests indicate taste bud size is comparable between the non-pregnant and pregnant females (p=0.7792, Figure 6A). In terms of epithelial thickness, there is no difference between the two groups (p=0.1621, Figure 6B).

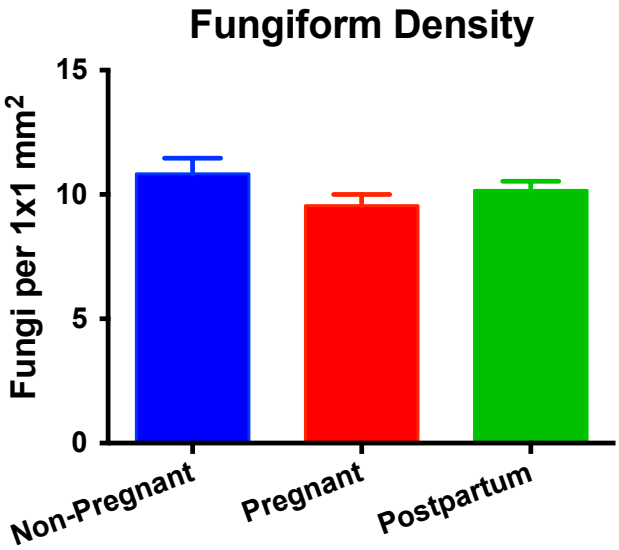

Figure 5. Fungiform density within 1mm x 1 mm square as counted from images of anterior tongues of non-pregnant, pregnant, and postpartum mice (n= 9-12). One-way ANOVA reveals no significant difference between treatment groups for fungiform density (p=0.2336).

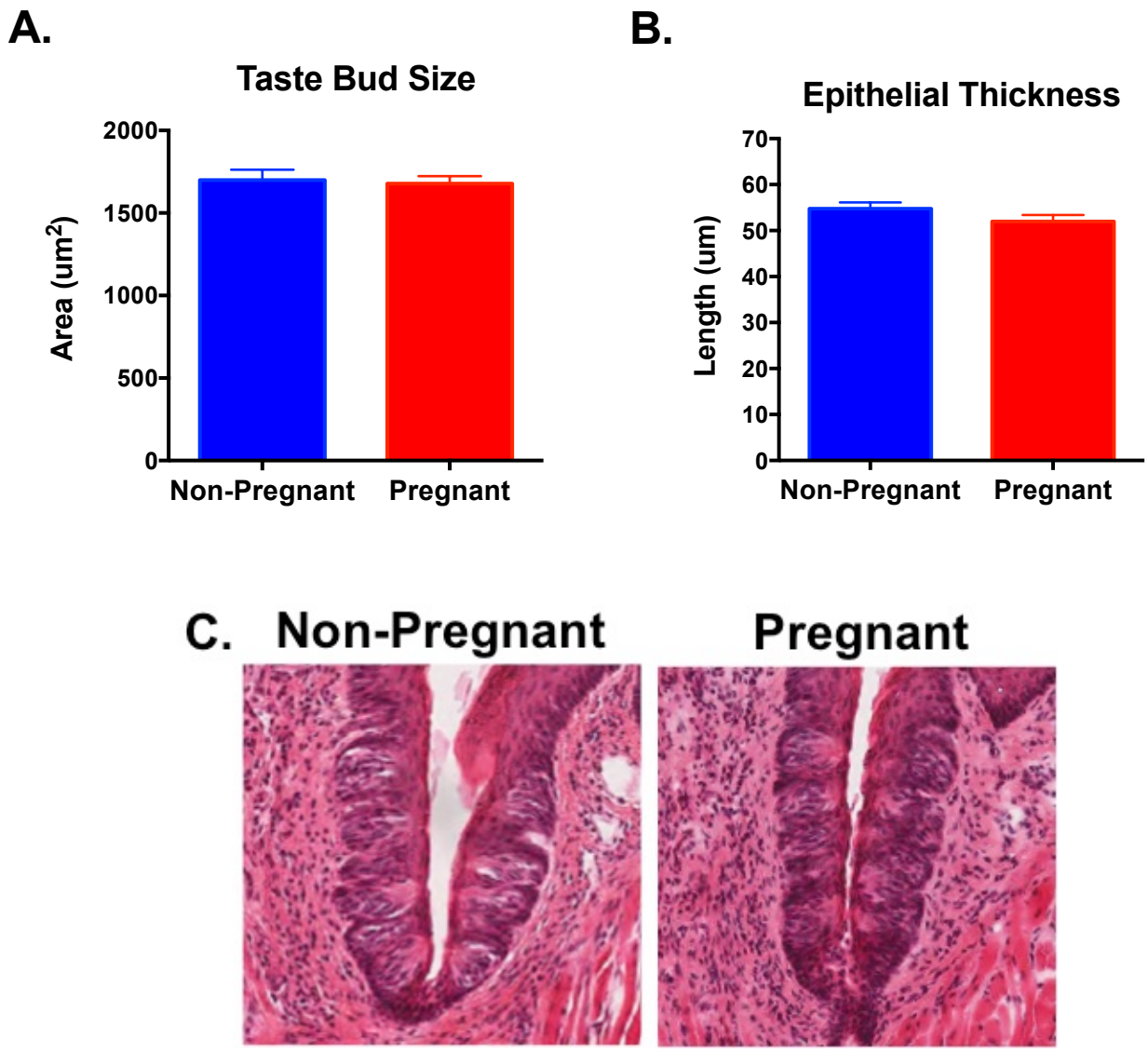

Figure 6. (A-B) Taste bud size and epithelial thickness (see supplement figure 1 for image example of epithelial measurement). (C) H&E staining of taste buds in the circumvallate papillae of females that were never mated (“non-pregnant”) and pregnant females Gt 18. Taste bud size and epithelial thicknesses were measured from tissue sections of the circumvallate papillae stained with hematoxylin and eosin (n=5 per treatment). T-tests were performed between the two groups with significant level $p<0.05$

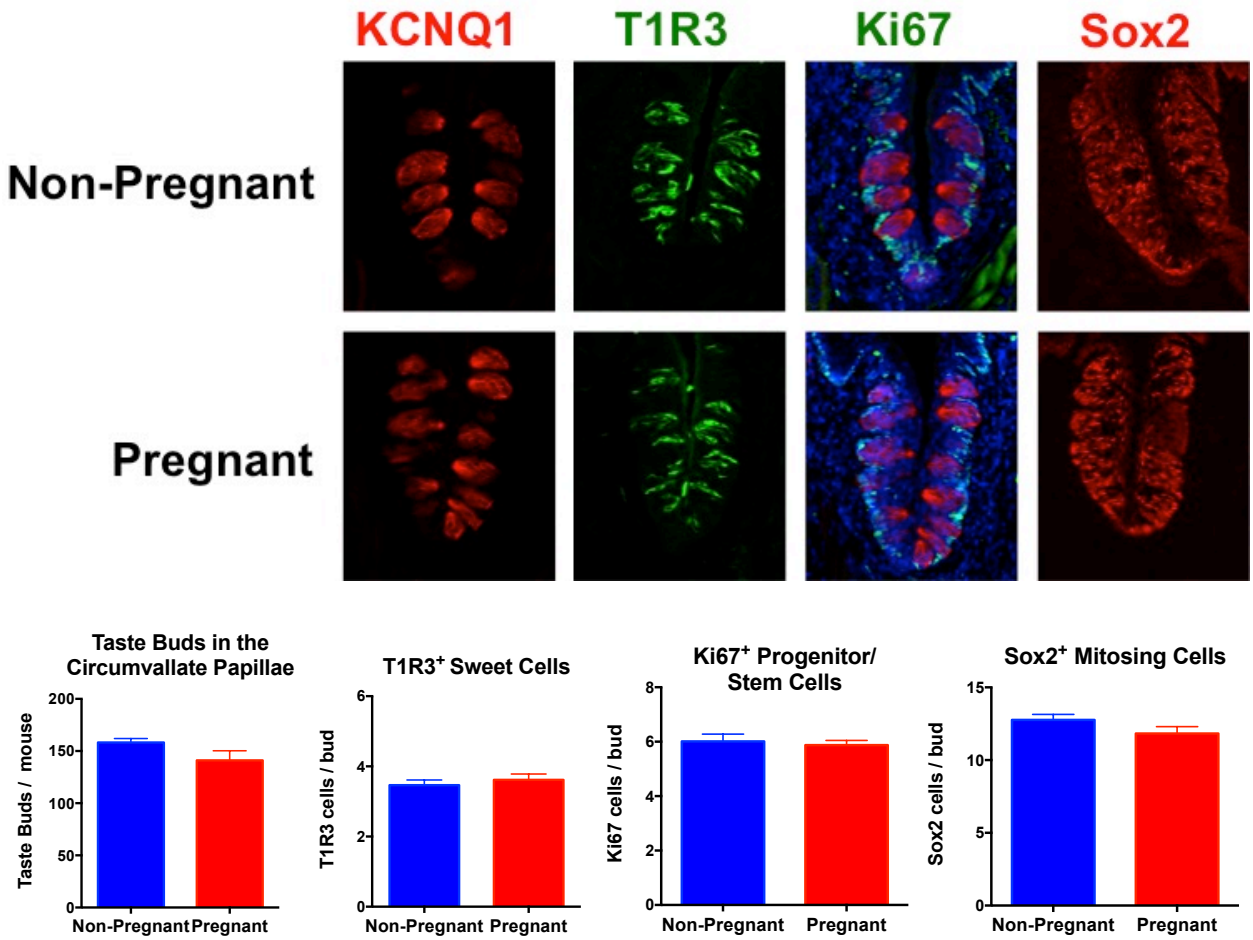

Figure 7. Number of taste buds and taste cells in the circumvallate papillae of non-pregnant and pregnant females Gt 18. The number of buds was counted from every 5th section of immunofluorescent stains for KCNQ1, a general taste cell stain. Cell counting for T1R3, Ki67, and Sox2 were performed from at least two taste buds from each section per circumvallate, and then repeated until cells were counted from 16 buds.

Poisson loglinear model indicated no change in the number of taste buds due to pregnancy (p=0.218, Figure 7). The effects of pregnancy on the number of classically defined types of taste cells were investigated using selective immunofluorescence staining for canonical taste cell markers (Table 2). Poisson loglinear model indicated no change in the number of T1R3 (p=0.423), Ki67 (p=0.799), and Sox2 (p=0.519) cells (Figure 7).

Discussion

Sucrose lick response during pregnancy is altered

Brief-access taste testing revealed sucrose lick responses to be significantly different from the non-pregnant and postpartum periods. During pregnancy, the sucrose lick response curve maximum was lower than prior to pregnancy and responses in the middle sucrose concentration range also appear lower than pre-pregnancy. When compared to postpartum, the pregnant mice have a lower maximum and greater relative EC50, suggesting that the curve is shifted to the right. The relative EC50 during pregnancy is greater than the postpartum period by 0.0211 M sucrose (Figure 3C), indicating that it takes a higher concentration of sucrose during pregnancy to produce the same lick response relative to postpartum. Studies of consumption and taste perception in pregnant compared to postpartum women indicate that women in their second trimester consumed significantly more sweet foods when compared to women in earlier stages of pregnancy (Bowen 1992). This suggests that women have the tendency to consume more sweet tasting foods as pregnancy develops.

Taste receptor expression is modulated across pregnancy

The sweet taste receptor is formed by a heterodimer of T1R2 and T1R3 subunits, while the receptor for umami (i.e. “savory”, the taste of amino acids) is formed by a heterodimer of T1R1 and T1R3 subunits. From brief-access Lickometer testing with sucrose we found sweet responses to be diminished during pregnancy compared to pre-pregnant and postpartum periods. Recent work suggests that receptor expression in taste buds is accurately predicted by receptor mRNA expression (Lipchock et al 2013). We expected to see a decrease in expression for T1R2 and T1R3 subunits, although

we found T1R2 and T1R3 expression to be unchanged during gestation days 6, 12, and 18 (Figure 4A). This suggests that sweet receptor gene expression may not contribute to the decrease sweet licking response that we see in brief-access Lickometer testing. T1R3 expression was decreased in postpartum compared to non-pregnant, although we did not detect a significant shift in sweet taste in the postpartum group. T1R1 gene expression was increased about 2-fold during Gt12, which could promote the taste perception of protein (Figure 4A). This is around the same time in which pregnant female mice will being to show significant signs of weight gain (Hau and Skovgaard Jensen 1987, von Goltsch et al 1980).

Tumor necrosis factor-α has been shown to decrease sweet sensitivity at the taste bud level (Feng et al 2012). Thus, we investigated the expression levels of TNFα across pregnancy. We found no change in TNFα expression levels suggesting that TNFα gene expression at least at the taste bud level during pregnancy may not contribute to the decrease in sweet licking response as indicated by brief-access Lickometer testing (Figure 4B). Because we used qPCR analysis on taste bud samples, these conclusions do not provide insight into the levels of TNFα circulating throughout the rest of the biological system. Bitter receptors T2R5 and T2R8 appear to be modulated throughout pregnancy. Selective stimulation of these bitter receptors can be performed using cycloheximide for T2R5 and denatonium for T2R8 (Adler et al 2000, Chandrashekar et al 2000, Matsunami et al 2000). T2R5 increased about 2-fold in early pregnancy at Gt 6 and then decreased at Gt 12. This increase in bitter receptor expression may serve a protective role to the mother fetus by allowing detection/ avoidance of potentially poisonous compounds, which tend to be bitter, at lower

concentrations. Retrospective pregnancy reports in women indicate that the development of food aversions is correlated with the onset of nausea (Bayley et al 2002). T2R8 was decreased during Gt12 and postpartum. In the gut, stimulation of T2R8 has been shown to regulate the secretion of ghrelin with functional effects on food intake and gastric emptying (Janssen et al 2011). Taste cells are capable of producing ghrelin, which has been shown to increase responses to sour and salt (Shin et al 2010). Whether T2R8 in the taste buds activate the release of ghrelin as it does in the gut requires further investigation.

Alpha epithelial sodium channels, α-ENaC, have been implicated in the detection of salty taste signaling (Simon 1992, Rehnberg et al 1993). The decreased level of ENaC expression in the taste buds during Gt 12 may be associated with a dampened response to salt taste or the need for higher salt stimulation to produce the same response as during pre-pregnancy (Figure 4C). In studies of gestating and lactating nulliparous Long-Evans rats using brief access Lickometer testing, pregnant rats showed an increased licking response to salt taste (Clarke and Bernstein 2001). In studies of adolescent women surveyed for their preference of salted or unsalted food items, during pregnancy the salt peanuts, chips, and crackers were preferred over the unsalted/ low-salted products (Skinner et al 1998).

Pregnancy impacts taste bud morphology

Taste bud size remained unchanged during in pregnant mice compared to non-pregnant females (that were never mated), while total taste bud numbers in the circumvallate papillae and epithelial tongue thickness was decreased (see supplement figure 1 for example of image epithelial measurement). Studies of the vomeronasal

sensory epithelium in female mice during pregnancy suggest enhanced neural progenitor-cell proliferation due to increased estrogen levels (Oboti et al 2015). Thus, we quantified the number of Sox2 and Ki67 positive cells in and around taste buds. Further analysis of taste cell morphology and make up will be performed with cell counting.

Studying taste during pregnancy as a way to understand feeding and other behaviors

Most of the research on taste during pregnancy has focused on changes in women's sensitivity to sweet, salty, sour, and bitter tastes. Findings of the effects of pregnancy on women's taste have been mixed and researchers speculate that biochemical and hormonal changes during pregnancy could act at the taste bud levels to drive these changes (Choo and Dando 2017). This idea is supported by studies demonstrating the role of estradiol in the control of food intake in females (Eckel 2011); however, whether estradiol can directly act on taste cell to modulate taste perception requires further study. If these changes in taste have a physiologic basis, then these findings should be observed across race, cultures and possibly species. This study reports changes in sweet taste in mice and other studies have shown increased licking responses to salt in rats (Clarke and Bernstein 2001), increased attraction to overripe and fermented fruits in gravid fruit flies (Hussain et al 2016a, Hussain et al 2016b). Studies have begun to investigate taste changes during pregnancy in various animal models, but more studies are needed.

In humans, studies suggest that the nausea and vomiting during pregnancy plays an important role in protecting the mother and embryo by causing women to avoid foods

that might be dangerous to themselves or the developing fetus (Flaxman and Sherman 2000). A better understanding of taste sensitivity, especially to bitter compounds, may provide insight as to how changes in taste during pregnancy is regulated to promote the health of mother and offspring. Studies of women who have had multiple pregnancies suggest that food consumption behavior in the first pregnancy had long lasting effects, as their food intake was lessened during their second and third pregnancies (Lim et al 2008). Another behavior related to bitter sensitivity includes cigarette smoking. Survey findings of women who smoked cigarettes prior to pregnancy suggest that they stopped smoking while pregnant because they developed an aversion to the taste and smell of tobacco smoke and 73% of the surveyed women resumed their smoking habits within 3 months after child birth (Pletsch and Kratz 2004). A deeper understanding of how taste changes during pregnancy, may contribute to the development of novel strategies to regulate food seeking behavior and smoking cessation.

Conclusions

The maternal diet is critical for proper development of the fetus and outcomes to the offspring (Kaiser et al 2009). The modulation of taste is essential for pregnant women to consume an altered balance of sugars, salt, and protein, due to the demands of the fetus, as well as providing enhanced protection from the consumption of toxins, via hypersensitivity to bitter taste. The idea of pregnancy impacting taste is not unusual given that changes in other sensory modalities during pregnancy and labor are widely reported (Cameron 2014, Hoekzema et al 2017). The morphometric analysis of taste buds during pregnancy as well as gene expression of the taste buds have remained

unexplored. Thus, the aim of this study was to survey brief-access taste responses in a longitudinal study of mice before and across pregnancy and perform morphometric and gene expression analyses on taste buds from female mice before, during, and after pregnancy. Here, we show that sweet responses are diminished during pregnancy. The underlying mechanisms resulting in altered sweet taste responses during pregnancy remain to be clarified, but we speculate that the decrease in taste bud number in the circumvallate papillae may precipitate changes in taste response. Our data indicate the gene expression of sweet taste receptor subunits T1R2 was unchanged during pregnancy and T1R3 was decreased in postpartum. The signaling of sweet taste is comprised of a network, not a single pathway. The act of gustation requires a taste stimulus to activate receptors found at the taste cell membrane, which then activates cellular signaling pathways to eventually transduce the signal to the brain to be perceived as gustation. Based on our current findings, further investigation into other transcripts involved in sweet signal transduction is warranted.

Supplement

		Gene of Interest							
Timepooint	**vs Timepoint**	**PLCβ2**	**T1R1**	**T1R2**	**T1R3**	**TNFα**	**T2R5**	**T2R8**	**ENaC**
Not Pg	**Gt 6**	ns	ns	ns	ns	ns	***	ns	ns
Not Pg	**Gt 12**	ns	****	ns	ns	ns	*	*	***
Not Pg	**Gt 18**	ns	ns	ns	ns	ns	ns	ns	ns
Not Pg	**Post**	ns	ns	ns	***	ns	ns	**	**
Gt 6	**Gt 12**	ns	**	ns	ns	ns	****	*	***
Gt 6	**Gt 18**	ns	ns	*	ns	ns	****	ns	ns
Gt 6	**Post**	ns	ns	ns	**	ns	****	**	*
Gt 12	**Gt 18**	ns	****	ns	ns	ns	ns	ns	****
Gt 12	**Post**	*	****	ns	*	ns	ns	ns	ns
Gt 18	**Post**	***	ns	ns	ns	ns	ns	ns	***
	ANOVA p=	0.0013	0.0234	0.0001	0.0001	0.1211	<0.0001	0.0004	<0.0001

Supplement Table 1. Taste bud samples from not-pregnant, pregnant, and postpartum females were analyzed with qPCR. One-way ANOVA was performed for each gene of interest with post-hoc Tukey when $p<0.05$. This table lists the multiple comparisons that were analyze, the resultant p values from one-way ANOVA, and the results of the comparisons for each gene.

Supplement Figure 1. Sample image of where epithelial thickness was measured. Typically, thickness was measured from both the left and right sides of the circumvallate papillae and was measure across a representative epithelial area with consistent thickness (i.e. not measured across a bump or tissue folded during handling).

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CHAPTER 4

Maternal Obesity Regulates Sweet Response and Receptor Gene Expression in Taste Buds of Offspring

Abstract

Maternal body mass index and gestational weight gain predict future over-weight or obese status in children and adolescents. In both rodents and non-human primates, maternal obesity predicts a preference for palatable foods in the offspring, suggesting an increased preference for foods rich in fat, sugar, and/or salt. In this study, we used C57BL/6J mice to investigate whether the underlying basis for an increase in palatable food consumption in the offspring of maternally obese mice is due to a change in taste. Mice were fed a normal control (NC) or high fat diet (HFD) before and during gestation and lactation, with all offspring subsequently maintained on control diet after weaning; thus, the only experience with HFD for the offspring was through maternal exposure during early development. The adult male offspring of maternal HFD treatment at 8 weeks old were slightly lower in body weight than controls while female body weight was similar between treatments. The adult offspring of both sexes show similar blood glucose levels and baseline water and chow intake at adult age. Taste response was assessed in offspring after reaching maturity, using brief-access taste testing. The offspring of maternal obesity showed an enhanced response to sucrose ($F_{4,321}$=2.008, p=0.0932). We hypothesize that this results from changes in the taste bud expression profile for sweet taste receptors. These same offspring had increased expression for subunits T1R2 (p=0.0321) and T1R3 (p=0.0005) that form the sweet receptor

heterodimer and showed increased consumption of 0.1 M sucrose, 1.0 mM sucralose, and high-fat diet relative to their lean control counterparts. The results indicate that behavioral changes in the adult offspring induced by maternal obesity correlate with increased expression of sweet taste receptors in the taste buds, which may drive the increased preference for palatable foods reported in offspring of maternally obese mice.

Introduction

Obesity is a worldwide chronic health problem with sizeable healthcare cost outcomes. A number of factors contribute to the increasing obesity rates such as genetics, a sedentary lifestyle, and ease of access to inexpensive calorie dense foods. Obesity is increasing at a higher rate in women than men (Kelly et al 2008, Wang et al 2008) and is also increasing in children (Adair 2008, Flegal et al 2012). In the United States, half of women of childbearing age are overweight or obese (Vahratian 2009). Considering that maternal obesity puts the child at an increased risk for childhood and adulthood obesity (Catalano and Ehrenberg 2006, Catalano et al 2009, Drake and Reynolds 2010), this could mean that future generations will continue to be put at an increased health risks associated with obesity due to *in utero* exposure to maternal obesity. The phenomenon of events *in utero* having long-term influences on disease risk later in life is known as 'early life programming'. Maternal obesity and overnutrition are now recognized as programming factors, which has led to the 'developmental overnutrition hypothesis'. The increased nutrients can lead to permanent changes in offspring metabolism, behavior and appetite regulation with the propensity for developing obesity, metabolic, and behavioral problems later in adult life (Drake and Reynolds 2010, Alfaradhi and Ozanne 2011, Li et al 2011).

The maternal diet may encourage offspring to develop taste preferences that mirror cues from the surrounding environment. Studies of maternal HFD have mostly focused on the detrimental effects to the offspring when switched to a control diet either at weaning or adulthood; however, evidence suggests that the offspring of maternal HFD that stay on the same HFD have decrease plasma triglycerides and improved

endothelial function compared to littermates that were switched a control diet (Khan et al 2004). This suggests that the maternal diet provides adaptive cues to the developing offspring.

A study in rainbow trout suggests that early diet exposure can program the sensory perception pathways that guide feeding behavior later in life. Rainbow trout normally fed with fish-based feed will show poor growth if switched to a plant-based feed. Balasubramanian et al (2016) investigated whether a brief early exposure to plant-based feed during the first-feeding period, when trout are first able to consume exogenous feed, would nutritionally program and prepare the trout for plant-based feed later (seven months later) in life. The trout with the early exposure showed improved growth and feed utilization compared to trout that had only ever received the meat-based feed. These improvements were associated with up regulation in sensory perception pathways and Tas1R2 receptors in the brains of the plant-based early exposure group (Balasubramanian et al 2016). The early exposure to plant-based feed may alter sensory perception pathways to facilitate acceptance of the same diet later in life.

Maternal obesity predisposes offspring to diet-induced obesity

Rodent studies of maternal obesity and/or HFD during gestation and lactation demonstrate that the offspring, when challenged with HFD after weaning, are predisposed to greater increases in adiposity, poor glycemic control, and metabolic dysregulation compared to offspring of lean dams fed normal chow (Benkalfat et al 2011, Howie et al 2009, Parente et al 2008). This propensity for diet-induced obesity in the offspring of obese dams has been linked with alterations to the reward system and

the hypothalamus (Page et al 2009, Rajia et al 2010), suggesting that regulation of reward-related feeding is affected in this model (Dietrich et al 2012, Leinninger et al 2011). Dysregulation of the reward system may contribute to the propensity to develop diet-induced obesity (Blum et al 2012, Volkow et al 2008). In obesity, reward signaling in relation to feeding is altered as suggested by human (Batterink et al 2010, Burger and Stice 2011, Stoeckel et al 2008) and rodent studies (Finger et al 2012, Johnson and Kenny 2010). Taken together, maternal obesity in early life exposure may contribute to the diet induced obesity of the offspring during exposure to a highly palatable high fat diet as adults (Penfold and Ozanne 2015).

Maternal HFD offspring have increased preferences for fat and sweet taste

One explanation for obesity is the influence of early-life nutrition on the taste system of the offspring. When male and female offspring are presented with HFD at weaning age, the offspring of HFD dams quickly overconsume and become obese sooner than offspring of dams maintained on control diet (Tamashiro et al 2009). At weaning, both male and female offspring of HFD dams tend to be heavier than the controls, but after being weaned onto control diet their weights become comparable to controls at adulthood. When offspring are studied at adult age of 11-12 weeks, where there is no significant difference in body weight, the differences between the two groups emerge once the animals are presented with the palatable high-fat diet – females of HFD dams consume significantly more HFD than the controls (Treesukosol et al 2014). These same female offspring of HFD dams also had higher preferences for corn oil than the controls. Overall, current evidence suggests that weight gain and obesity in offspring of HFD dams may be due in part to changes in taste.

Does fetal exposure to fats result in stimulus-induced chemosensory plasticity?

Maternal obesity or HFD will cause the offspring to overconsume high-fat and high-sugar foods compared to their lean counterparts (Bayol et al 2007, Ong and Muhlhausler 2014, Tamashiro et al 2009, Teegarden et al 2009, Walker et al 2008), which can increase the risk for obesity, as these foods are calorie dense. This increase in consumption is associated with an increased preference for sucrose, fat, or palatable foods (Chang at al 2008, Vucetic et al 2010, Naef et al 2011, Ong and Muhlhausler 2011, Teegarden et al 2009). Preferences for fat and sweet are positively correlated with overweight and obese status in adolescents (Lanfer et al 2011). Epidemiological and animal studies reveal that a maternal HFD is highly predictive of a preference for fatty foods in the offspring (Sullivan et al 2011). Dietary fat intake is a strong predictor of obesity in adult women who were once lean, but only if they had a family history of obesity. This suggests that a preference for fats precedes the development of obesity (Reed et al 1997). In nonhuman primate studies of *Macaca Fuscata*, dams fed HFD for 2-7 years and through gestation/ lactation produced offspring that overconsumed HFD and sucrose relative to the controls at weaning (Rivera et al 2015). The objective of this project was to investigate whether the taste system may also be altered in this model of maternal obesity with subsequent weaning onto normal chow into adulthood.

Perinatal flavor programming

In neonates, sweet, umami, and low concentrations of salty substances are innately preferred, whereas bitter and sour substances are rejected. Studies show that exposure to certain taste stimuli during infancy and early childhood can modify these innate tendencies and alter dietary preferences in children years later (Johnson et al

1991, Kern et al 1993, Liem and Mennella 2002, Mennella and Beauchamp 2002, Mennella 2014). This influence on infants is referred to as “flavor programming” or “flavor imprinting” (Beauchamp and Mennella 2009, 2011). Studies by Mennella et al (2001) reveal that mothers fed carrot juice during their third trimester pass carrot flavor acceptance to their offspring. Follow-up studies showed that babies of mothers who ate more fruit during lactation were more likely to accept fruits than babies fed formula; though vegetable consumption did not have the same effect (Forestell and Mennella 2007). Another study investigated formula-fed infants (less than a month old) given either a cow milk-based formula control or hydrolyzed protein hydrolysate formula, which had relatively more bitter, sour, and savory tastes, for up to 8 months. The infants were then tested for their acceptance and intake of a savory or plain broth. The infants that consume the treatment formula showed greater acceptance and increased consumption of the savory broth relative to plain (Mennella and Castor 2012).

There is evidence linking the parental diet and nutritional status to metabolic and phenotypic traits in offspring (Rando and Simmons 2015), suggesting that the perinatal period provides a window for nutritional intervention that can have lifelong effects on dietary preference and eventual overall health in the offspring. However, whether maternal food ingestion during pregnancy directly influences food preferences in the offspring remains controversial. The magnitude of this early exposure on the development of food preferences that last into adulthood requires further investigation. In humans, several factors could influence food acceptance such as repeated exposure (Sullivan and Birch 1994), personal experiences, cultural adaptations, and perceived health benefits (Scaglioni S et al 2008).

Does maternal HFD exposure affect offspring taste system?

The present experiment was designed to examine the effects of maternal HFD exposure on taste and palatable food preference in the adult offspring. Given the ability to control the post-weaning diet in offspring of HFD dams, mice provide a useful model for studying whether maternal HFD promotes long-term effects on the offspring taste system to ultimately drive feeding behavior. We hypothesized that maternal HFD exposure increases palatable food preferences by altering basic taste responses in the adult offspring.

Methods

Animals

In-house bred virgin C57BL/6 female mice were randomly assigned to one of two diets *ad libitum* at 8 weeks of age (n=6 per group): (1) standard normal chow (NC, 18% kcal from fat) and (2) high-fat diet (HFD, 58.4% kcal from fat, Teklad Diets). See Table 1 for diet details and for primary data sheet details, see Appendix – High Fat Diet and Normal Chow data sheets. Females were maintained on their respective diets during the pre-mating period (starting at 8 weeks until 13 weeks of age). After 5 weeks on their respective diets, the females were mated in parallel (i.e. a NC female was mated at the same time that a HFD female was mated) with healthy lean males maintained on NC. Since females do not reliably consume HFD, relative to males, only females on HFD that gained at least 25% body weight from baseline were used for mating and continued on in the experiments. During mating, females were placed in the male cages containing NC and water. Day 1 of pregnancy was determined by detection of

copulatory plug and females were then returned to their home cages and maintained on their assigned diet treatment. Upon mating, minimal measurements of the female mice were taken to avoid adding stress from handling. On postnatal day 3 (P3), litter size thresholds were set to 5-8 pups with equal numbers of males and females where possible, to prevent any effects of under or overnutrition. Pups were nursed freely and all groups were weaned at 3 weeks onto NC, provided *ad libitum*. At 7 weeks the offspring were single housed and given a week to acclimatize before measurements and testing (Figure 1). Adult offspring of both sexes were studied. All animal work was approved by the Institutional Animal Care and Use Committee at Cornell University.

	NC			HFD		
Nutrients	g	Kcal	%Kcal	g	Kcal	%Kcal
Fat	6.2	55.8	18.0	20.4	315.4	58.4
Protein	18.6	74.4	24.0	36.1	81.0	15.0
Carbohydrate	44.2	179.8	58.0	35.2	143.6	26.6
Total		310	100		540	100
Kcal/g		3.1			5.4	

Table 1. Content of fat, protein, and carbohydrate in the normal control diet (NC) and high-fat diet (HFD) used for the maternal HFD treatment during gestation and lactation. Values are as reported in data sheets provided by manufacturer. Grams are per 100 g of diet.

Adult offspring measurements

At weaning, female and male offspring were fed NC for 5 weeks (i.e. until 8 weeks of age). The adult offspring were weighed and baseline measurements of water and NC intake over were recorded over 48 hours. Before any behavior testing mice were diet fasted in the morning for 4 hours and circulating blood glucose levels from tail blood was measured using a OneTouch UltraMini glucose meter.

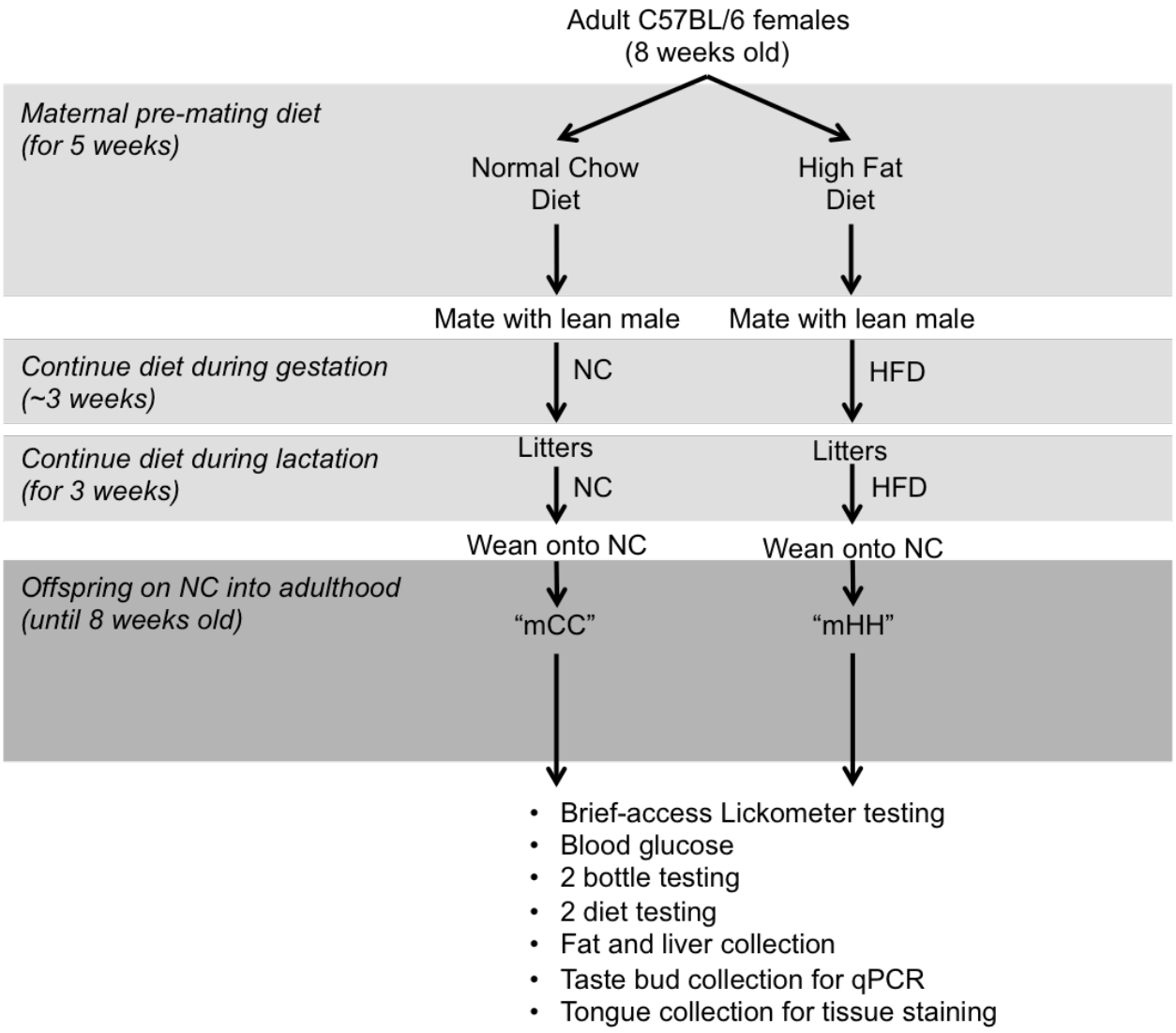


Figure 1. Schematic of study design. For the maternal treatment, 8 week old females were fed normal chow (NC) or high-fat diet (HFD) for 5 weeks pre-conception and throughout the gestation/ lactation period. All offspring were then weaned onto regular chow and examined as adults, at 8 weeks of age giving them time to reach full maturity. Acronyms "mCC" for controls and "mHH" for the treatment group are used throughout this manuscript.

Sweet Taste Behavior Assay – Brief-Access Lickometry

Taste responses (i.e. taste-related affective potency of the stimuli) were measured using a brief-access Davis Lickometer, which minimizes confounding factors such as appetite and post-ingestive effects. This method quantifies immediate lick responses to

extremely small volumes of sapid solutions and the training and testing schedule was adapted from Glendinning et al 2002 and Glendinning et al 2005. Training and testing were conducted under simulated dark cycle conditions (under red lights). To acclimate mice to the Lickometer testing chamber, mice were partially water restricted by providing 1 mL of water for 23.5 hour and then placed in the Lickometer and trained to lick from an available spout containing water for 30 minutes. For two additional days the mice were partially water restricted and placed in the Lickometer for 45 minutes each day with the full Lickometer functioning (again only water was in the bottles). Before testing, mice were partially water and food restricted by providing 1 mL of water and 1 g of normal chow for 23.5 hours prior to tastant training. For sweet testing, a range of sucrose (0, 0.03, 0.1, 0.2, 0.3, 0.6, 1.0 M) concentrations was used. The presentations were randomized in blocks so that every concentration was presented once before being repeated again. Once the mouse initiated licking on the presented bottle, the timer started for 5 seconds and then the shutter closed. Each test session lasted no more than one hour, during which the mouse could initiate up to 5 blocks of 7 concentrations (i.e. 35 total presentations). Linoleic acid (tastant for fat tested at 0, 0.003, 0.01, 0.02, 0.05, 0.1, and 0.3 M) and mono-sodium glutamate (tastant for umami tested at 0, 0.003, 0.01, 0.02, 0.05, 0.1, and 0.3 M) responses were tested sucrose testing following the same protocol (Supplement 3).

All Lickometer data were downloaded as .csv files and imported into Excel for further data analysis. Lick responses were normalized, fit to nonlinear variable slope concentration-response curves, and compared using extra sum-of-squares F test. The number of licks for each concentration was averaged within each mouse. These

averages were then divided by the maximal lick rate and subtracted from the minimum rate within each mouse yielding the standardized lick ratio. A lick ratio of 0.0 indicates that the sucrose concentration elicited minimal licking over water, whereas a value of 1.0 indicates maximal licking; thus, controlling for individual differences in local lick rate for each mouse. Tastant concentration–lick ratio response curves were fitted to the mean data for each group using a classical four parameter logistic sigmoidal dose–response equation in the nonlinear regression suite of GraphPad Prism (v5.0). Because we wanted to examine if there were any differences in the maximum and minimum responses, all four parameters remained unconstrained (i.e. bottom was not constrained to 0 and top was not constrained to 1).

Two Bottle Testing – Sucrose and Sucralose

Adult offspring were single housed and provided NC *ad libitum*. Mice were trained to consume water from two bottles for 48 hours. First, the mice were tested with one bottle of water and one bottle of sucrose (0.001, 0.02, 0.1 M sucrose in separate testing days) and then with one bottle of water and one bottle of sucralose (0.1, 0.3, 1.0 mM sucralose on separate testing days). Mice were given simultaneous access to the two bottles and consumption was measured over 24 hours. Bottle order was random and was switched after 48 hours. Sucrose and sucralose preferences were calculated as a percentage of sucrose intake divided by total fluid intake. For more details, see Appendix – SOP Two Bottle Testing.

Diet Preference and Intake

Mice were single housed and trained to consume NC from duel hoppers for 48 hours. Then mice were presented with NC in one hopper and HFD in other for 24 hours

over three test days. After every 24 hours, the diet sides were swapped. Intake of NC, HFD, and mouse body weight were measured. The intake from the final two days were summed over a 48 hour period and analyzed, while day one was excluded to allow animals to adapt to the novel diet. HFD preference was calculated as a percentage of HFD intake divided by total food intake by weight. Two diet testing methods were adapted from Vucetic et al 2010 and Carlin et al 2013. For more details, see Appendix – SOP Diet Preference Protocol.

Taste Bud Isolation and RNA Extraction

Taste buds from the circumvallate papillae were isolated from mice at ~10 weeks old after Lickometer testing and one week wash out of NC and water *ad libitum*. Mouse tongues were freshly excised following euthanization with CO_2 and cervical dislocation. The isolated tongue was immediately immersed and rinsed in Normal Tyrode's solution (135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM MgCl, 5 mM $NaHCO_3$, 10 mM HEPES, 10 mM Glucose, 10 mM Sodium Pyruvate; pH 7.4). The sublingual epithelium surrounding the circumvallate papillae was injected with enzyme cocktail and then incubated in Ca^{2+} free Tyrode's solution (135 mM NaCl, 5 mM KCl, 20mM EGTA, 10 mM HEPES, 5 mM BAPTA (1,2-Bis (2-aminophenoxy) ethane-N, N, N, N-tetraacetic acid tetrapotassium salt), 10 mM Glucose, 10 mM Sodium Pyruvate; pH 7.4) for 15 minutes at room temperature (Dispase II 2.5 mg/ml, Collagenase A 1mg/ml, Elastase 0.25 mg/ml, and DNaseI 0.5mg/ml in Normal Tyrode's solution). The top epithelium was carefully peeled away from the tongue and individual taste buds were collected using a glass fired polished micro pipette with coated in 0.2% PVP to prevent cells from sticking to the glass. Additionally, a piece of the epithelium posterior to the circumvallate papillae

was cut out after taste bud collection, known as the “non-taste” area was collected as a non-chemosensory control epithelial tissue, and processed in parallel. All samples were immediately lysed and processed for RNA extraction.

RNA extraction, reverse transcription, and determination of gene expression

Total RNA was extracted using Absolutely RNA Nanoprep Kits for taste samples and RNA Microprep Kits for non-taste samples (Agilent, Stratagene) and used as template for cDNA synthesis with qScript cDNA SuperMix (Quanta Bio, Beverly, MA). Quantitative real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) was run on a QuantStudio 6 Flex Real-Time PCR System (Thermo). PLCβ2 enrichment over non-taste samples was used as a positive control for taste cells. Relative quantification was performed in triplicates using QuantStudio PCR Software, based on the $2^{-\Delta\Delta Ct}$ method. Beta-Actin was used as the endogenous housekeeping gene for normalization of genes of interested (Table 2). To control for false positives, a non-template control was run for each template and primer pair. The treatment groups were compared using t-test, with $p>0.05$ as significant.

Protein	Gene	Forward 5'-3'	Reverse 5'-3'	Product size (bp)
β-actin	*Actb*	caccctgtgctgctcacc	gcacgatttccctctcag	328
T1R2	*Tas1r2*	aagcatcgcctcctactcc	ggctggcaactcttagaacac	114
T1R3	*Tas1r3*	gaagcatccagatgacttca	gggaacagaaggacactgag	283
Gα14	*Gna14*	attagctacttcccagagtacaca	gctcagatcaccctctgtct	256
PLCβ2	*Plcb2*	gagcaaatcgccaagatgat	ccttgtctgtggtgaccttg	163
TrpM5	*Trpm5*	gtctggaatcacaggccaac	gttgatgtgccccaaaaact	234
T1R1	*Tas1r1*	ctggaatggacctgaatggac	agcagcagtggtgggaac	185
CD36	*Cd36*	ggccaagctattgcgacatg	ccgaacacagcgtagatagac	124
GPR120	*Gpr120*	ctggggctcatctttgtcgt	acgacgagcactagagggat	155
T2R5	*Tas2r105*	gaatcatagaaacaggacctcg	ctttacaaaggcttgctttagc	406
T2R8	*Tas2r108*	ttctgatttcagccctcacc	ccaaaagctggtcctgtttc	245

Table 2. Genes of interest and their corresponding primer sequences.

Postmortem and tissue collection procedures

One hour prior to euthanization, mice were injected with serotonin 5-HTP (2 mg / 25 g body weight) to enhance immunofluorescence stain for 5HT+ taste cells. Mice were then humanely euthanized with CO_2 and cervical dislocation. Tongues were excised and rinsed in PBS. The circumvallate papillae was carefully isolated with a sterile razor and fixed in 4% PFA at 4°C for one hour, cryoprotected in 30% sucrose overnight, and then embedded in OCT. The anterior two-thirds of the mouse tongue was fixed in 4% PFA for at least 24 hours before staining with 1% methylene blue for fungiform density analysis. Perigonadal fat pads were dissected and weighed. Livers were collected and immediately fixed in 10% neutral buffered formalin fixative for staining with oil red O. Liver sections were imaged using an Aperio CS2 at 40x magnification (Leica).

Immunofluorescence and morphometric analyses

The 4% PFA fixed tissue was sectioned at 10 microns and stained with hematoxylin and eosin (H&E) or antibodies for immunofluorescence (Table 3). Images were taken using an Olympus IX-71 microscope with a Hamamatsu Orca Flash 4.0 camera. For immunofluorescence, tissue was incubated at room temperature with blocking solution (2% BSA, 2% donkey serum, 0.3% Triton) for at least 3 hours, and then incubated at 4°C overnight with primary antibody. AlexaFluor 488, 594, 647-conjugated secondary antibodies raised in donkey against rabbit, goat, or rat (1:1000 dilution) for 2 hours of secondary antibody incubations. In order to obtain an unbiased count from taste buds in the circumvallate, every 8th section was used for quantification. The percentage of cells of interest was determined by cell counting. In

brief, random taste buds from the left and right sides of the circumvallate were chosen, with a minimum of 10 buds per mouse (n=4 per sex for each treatment). Slides were mounted using DAPI Fluoromount-G (SothernBiotech).

Antigen	Host	Vender	Dilution
NTPDase2	rabbit	J. Sévigny at Université Laval, Quebec	1:1000
PLCβ2	rabbit	Santa Cruz Biotechnology	1:1000
IP3R3	goat	Santa Cruz Biotechnology	1:1000
T1R2	goat	Santa Cruz Biotechnology	1:200
T1R3	goat	Santa Cruz Biotechnology	1:1000
KCNQ1	goat	Santa Cruz Biotechnology	1:1000
5HT	rat	Millipore	1:1000
Gustducin	rabbit	Santa Cruz Biotechnology	1:1000
Ki67	rabbit	Thermo	1:1000
Sox2	goat	Santa Cruz Biotechnology	1:1000

Table 3. Primary antibodies used for immunofluorescence analysis

Fungiform Density

The anterior two-thirds of the mouse tongue was excised and fixed in 4% PFA for at least 24 hours before staining with 1% methylene blue for 1 minute, rinsed with PBS, and then imaged under a light microscope. The fungiform papillae appear as lighter blue dots on a background of blue stained tongue epithelia and were counted using ImageJ. In a region of interest representing a 1x1 mm square.

Statistical Analysis

Data are presented as mean ± SEM. The effects of maternal HFD and offspring body weight, perigonadal adipose weight, blood glucose, chow intake, water intake, HFD preference, and HFD intake were analyzed using a two-way ANOVA with sex and maternal treatment groups as factors. Preference and intake results from two-bottle testing and gene expression data were analyzed using two-way ANOVA with maternal treatment and sucrose/ sucralose concentrations as factors. When a significant

interaction was identified, data were analyzed with post-hoc Tukey multiple comparisons. All analyses were carried out in GraphPad Prism (v5.0) and the probability of $P < 0.05$ was considered to be statistically significant.

Results

During the pre-mating period, NC females on averaged gained 10% ± 2.3 body weight from baseline, while the HFD females on average gained 33% ± 2.8 body weight from original (Figure 2). We studied the adult offspring starting at 8 weeks of age, after which we did not expect age to be a considerable contributor to changes in taste that we see (Shin et al 2012). We also expected maternal HFD to not have any effect on litter size (Ornellas et al 2013). Findings were stratified by sex because we found differences in sweet taste response in the Lickometer behavior data. There are differences in taste processing between males and females in both the periphery and the brain although the casual mechanisms require further investigation (Martin and Sollars 2017).

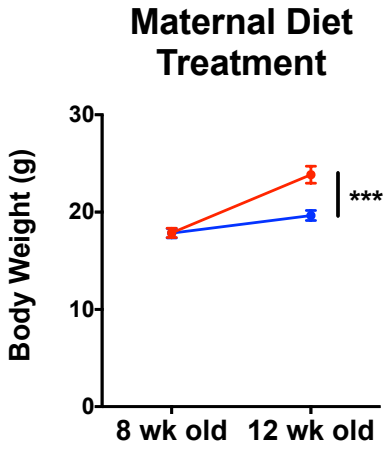
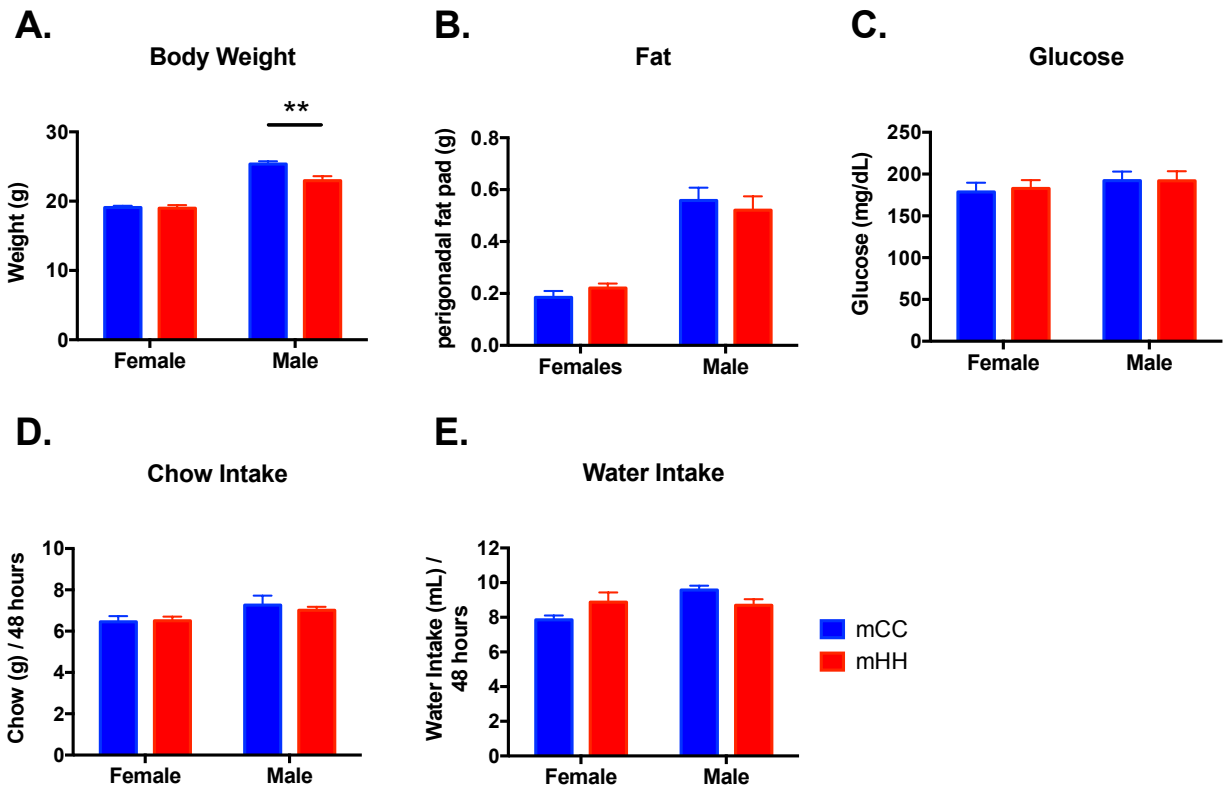


Figure 2. The final BW of the HFD females at time of mating (12 weeks old) was significantly difference from the NC females; Student's t-test, p=0.0002. (body weight was measured in n=5 females for each diet treatment).

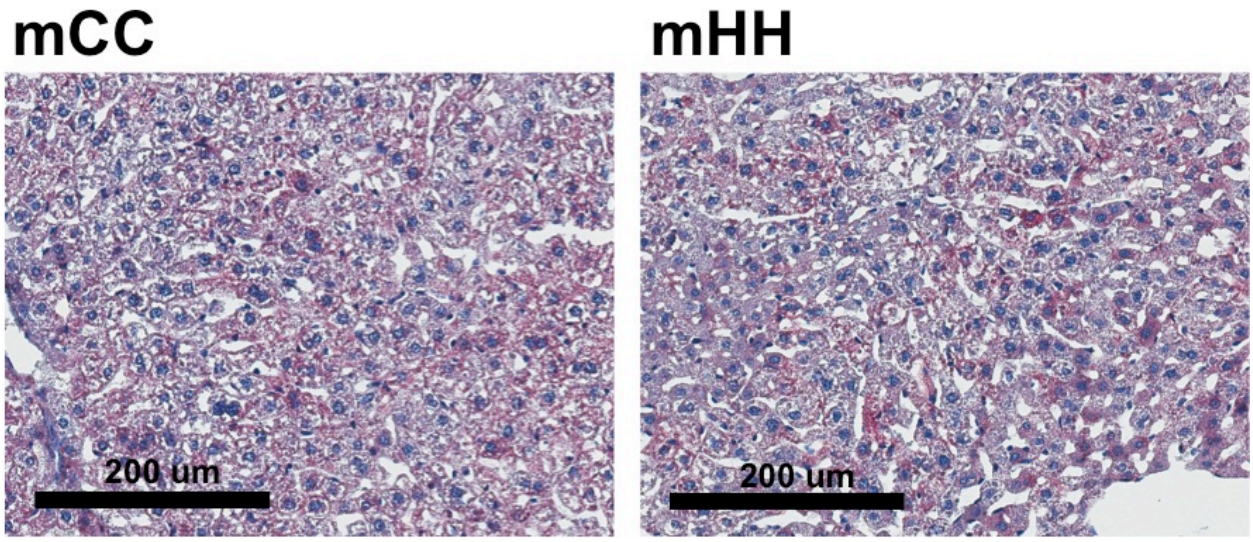
Adult offspring outcomes at 8 weeks of age

It is possible for adult offspring of HFD dams to have normal glucose tolerance and body compositions compared to their lean counterparts (Platt et al 2014). Two-way ANOVA showed an interaction of maternal treatment and sex ($F_{1,36}$=5.57, p=0.0238), while post-hoc Tukey analysis showed that mHH males exhibited a decrease in body weight compared to control males (Figure 3A). Maternal HFD exposure had no impact on perigonadal fat pad weight, blood glucose levels, and baseline chow and water intake between sexes (Figure 3B-E). Two-way ANOVA showed an interaction of maternal treatment and sex for water intake ($F_{1,36}$=6.834, p=0.0130), while post-hoc Tukey analysis revealed a significant difference between the mCC females and mCC males ($p<0.05$), but no difference for all other comparisons between treatments (Figure 3E). Oil Red O staining was performed to measure hepatic lipid accumulation as a sign of any underlying disruption to fat metabolism. Histological examination revealed lipid composition was unchanged between treatments (Figure 4).



	Group	Female		Male		
	n=	mCC	mHH	mCC	mHH	ANOVA
Body Weight (g)	9-11	19.09 ± 0.24	18.99 ± 0.44	25.35 ± 0.42	22.95 ± 0.65**	M x S, M, S
Perigonadal Fat (g)	7-11	0.185 ± 0.02	0.221 ± 0.01	0.558 ± 0.05	0.521 ± 0.05	S
Glucose	6-9	178.6 ± 11.2	182.8 ± 10.1	192.1 ± 11.0	192.0 ± 11.5	
Intake over 48 hours						
Chow (g)	9-11	6.456 ± 0.27	6.500 ± 0.21	7.264 ± 0.46	7.009 ± 0.17	S
Water (g)	9-11	7.856 ± 0.25	8.878 ± 0.55	9.573 ± 0.26	8.691 ± 0.35	M x S, S

Figure 3. Metabolic parameters in maternal NC and HFD offspring at 8 weeks of age with summary table below. Values are expressed as mean ± SEM. Data was analyzed by two-way ANOVA and post-hoc Tukey multiple comparisons test (when maternal diet x sex interaction was identified). M = maternal diet effect, S = sex effect, M x S = maternal diet and sex interaction. Significance shown here is based on comparisons between the maternal treatments within each sex; *p < 0.05, **p < 0.01.



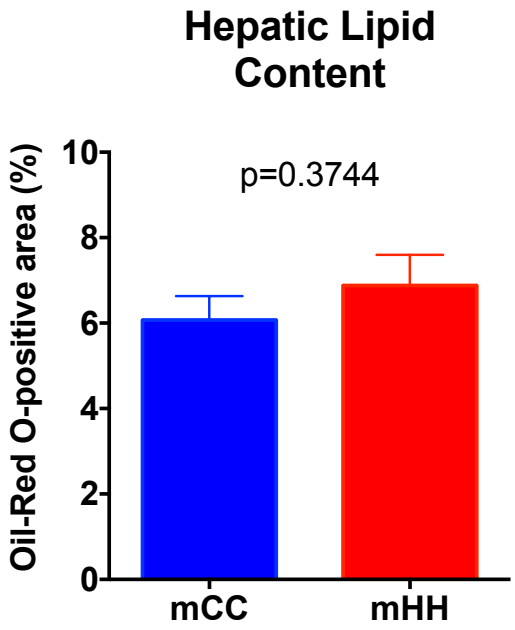


Figure 4. Liver sections were stained with Oil Red O for histological examination. Images were taken using 40x magnification. Images were analyzed using color deconvolution in ImageJ (unpaired t-test, n=6, 3 sections per mice was used in the analysis).

Long-term effect of maternal HFD on sucrose response and preference and intake in adult offspring

Brief-access sucrose lick responses were evaluated in the adult offspring at postnatal week 8-9 (Figure 5). Four parameter nonlinear regression analysis of the concentration-response curves for sucrose showed no significant different between the mCC and mHH groups when both sexes are analyzed together ($F_{4,321}$=2.008, p=0.0932).When stratified by sex, the female offspring of maternal HFD showed an increase lick response to sucrose compared to control females ($F_{4,167}$=2.844, p=0.0258), while there remained no difference in the males between maternal treatments ($F_{4,146}$=0.9026, p=0.4642). The maximum for mHH females (0.7936 ± 0.07232) was slightly lower than the controls (0.8262 ± 0.1073). The minimum in mHH females (0.03239 ± 0.06747) than controls (0.111 ± 0.05530). The Hill Slope was steeper in the control females (2.125 ± 1.025) than the mHH females (1.635 ± 0.6234). The relative EC50 for the mHH females (0.09662 ± 1.2541) was much lower than controls (0.2355 ± 1.2930, Figure 5A). Absolute EC50 values were not determined because the curves were not constrained to a minimum value of zero and maximum of one, even though the data was normalized.

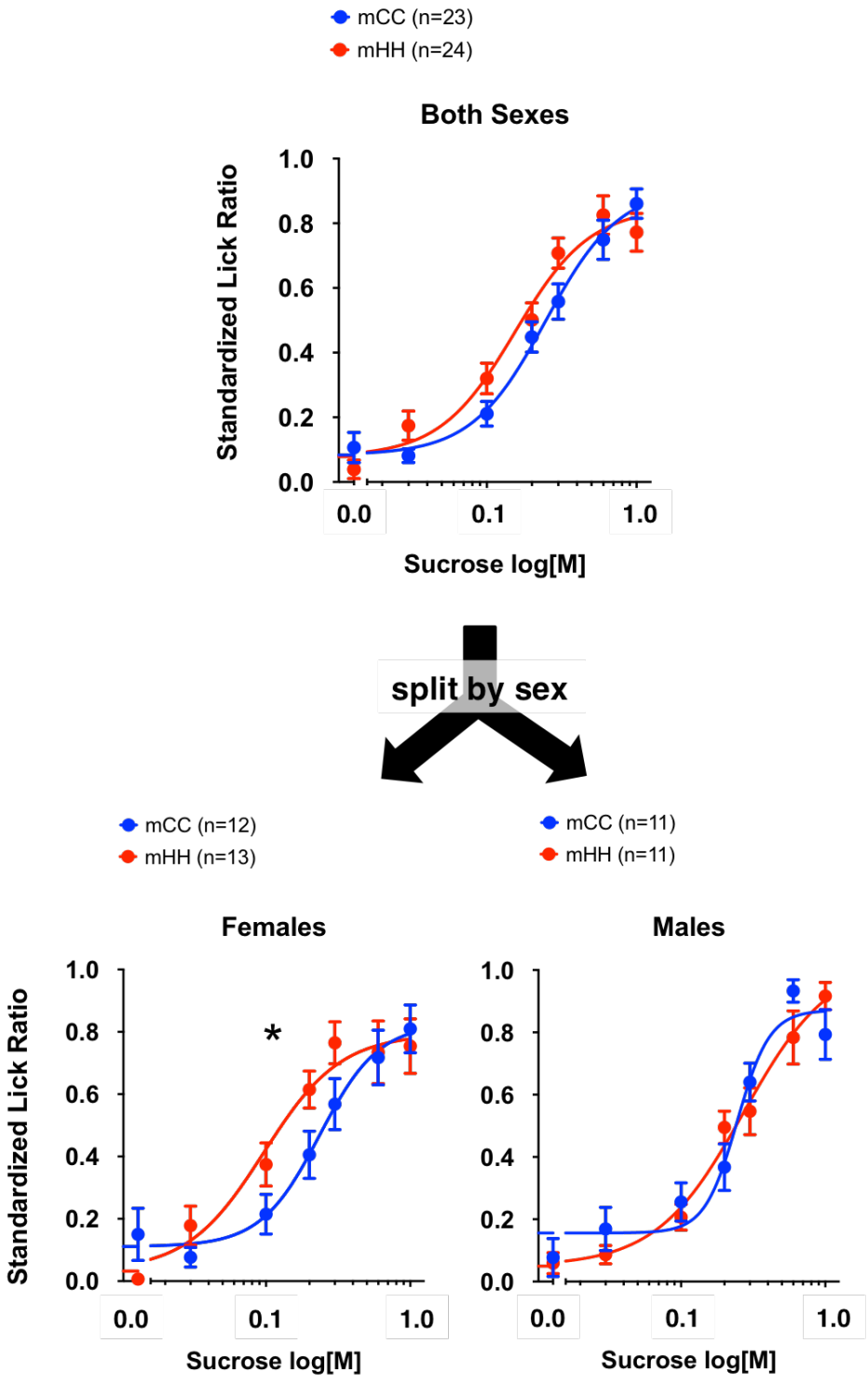


Figure 5. Adult females of mHH treatment group show increased licking response compared to female controls.

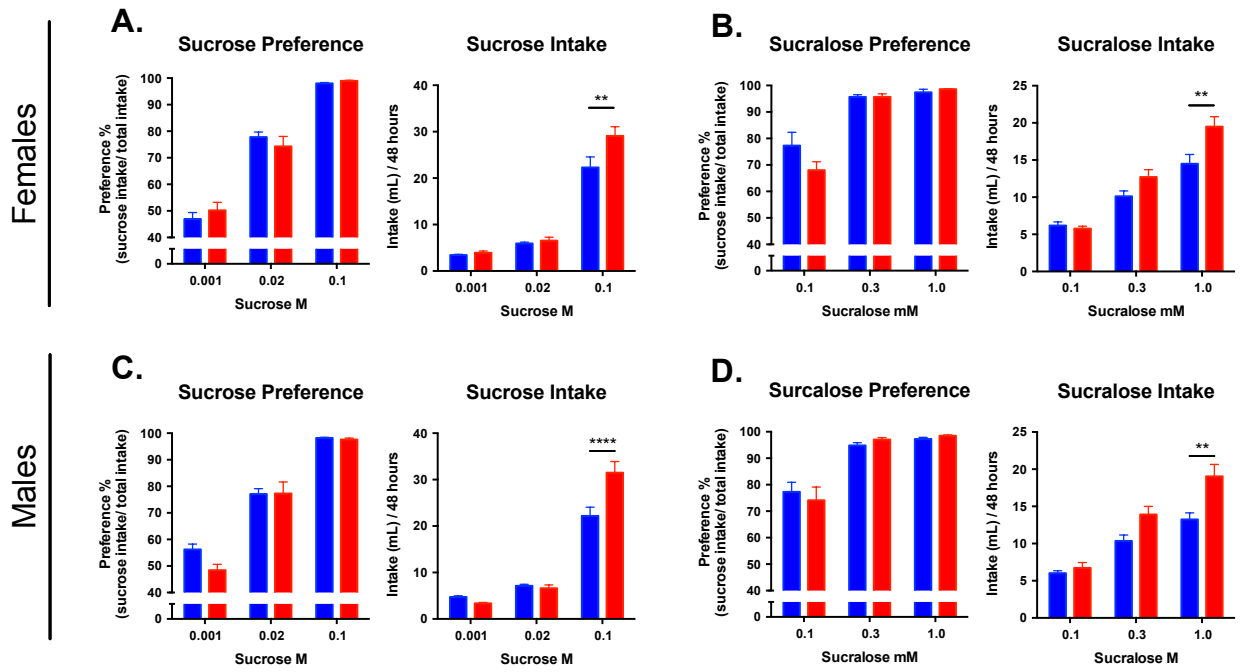


Figure 6. Two-bottle preference and intake for sucrose vs water and sucralose vs water by (A-B) adult females (n=9 per treatment) and (C-D) male offspring (n=11 per treatment). Data were analyzed by two-way ANOVA and post-hoc Tukey multiple comparisons (when maternal diet x sex interaction was identified): **/**** when P < 0.01/0.0001. Blue = offspring of dams fed NC; Red = offspring of dams fed HFD.

Sweet preference and intake, using sucrose and sucralose solutions, was measured over 48 hours with adult offspring week 8-9 (Figure6A-D). This was performed with a separate group of mice naïve to any previous behavior testing and had not been evaluated for brief-access sucrose responses. At adulthood there was no significant differences in sucrose and sucralose preference amongst the two maternal treatment groups. Two-way ANOVA revealed a significant interaction between maternal treatment and concentration for sucrose intake in females ($F_{2, 48}$=3.896, p=0.0270; Figure 6A) and in males ($F_{2, 60}$=10.64, p=0.0001; Figure 6C). Specifically, Tukey analysis indicated that mHH females (Figure 6A) and mHH males (Figure 6C) consume more 0.1M sucrose than to their respective controls. Similar to the findings of sucrose,

two-way ANOVA revealed a significant interaction between maternal treatment and concentration for sucralose intake in females ($F_{2, 48}$=4.266, p=0.0.0197; Figure 6B) and in males ($F_{2, 60}$=3.371, p=0.0410; Figure 6D). Specifically, Tukey analysis indicated that mHH females (Figure 6A) and mHH males (Figure 6C) consume more 1mM sucralose than to their respective controls.

Long-term effect of maternal HFD on high-fat diet preference and intake in the adult offspring

Preference and intake of high-fat diet was evaluated in the adult offspring at postnatal weeks 10-11 (Figure 7). Ordinary two-way ANOVA showed a significant interaction between maternal treatment and sex for HFD preference percentages (p=0.0286); however post-hoc Tukey multiple comparison revealed no significant differences between sex or treatment (Figure 7A). Concerning the total HFD consumed over 48 hours (Figure 7B), we detected a significant interaction between maternal treatment and sex ($F_{1,29}$= 4.623, p=0.0400). Specifically, Tukey analysis indicated that mHH females consume significantly more HFD relative to mCC females (p<0.05).

Long-term effect of maternal HFD on the expression of sweet taste signaling in the taste buds of the adult offspring

Two-way ANOVA showed a main effect of maternal treatment on gene expression of all sweet receptor and sweet signaling genes investigated (T1R2, T1R3, Gα-14, PLCβ2, TRPM5). We also found a main effect of sex on the gene expression of T1R2 (p=0.0091) and T1R3 (0.0010). A significant interaction between maternal treatment and sex was detected for PLCβ2 (p=0.0429). Tukey analysis indicated a significant increased in T1R2 (p<0.05) and T1R3 in mHH females (p<0.05) compared to

mCC females (Figure 8A). Similarly, T1R2 ($p<0.01$) and T1R3 ($p<0.001$) expression was increased in the mHH males relative to mCC males, in addition to increased gene expression of TRPM5 ($p<0.05$, Figure 8B). The mHH males presented increased gene expression for T1R2 ($p<0.0001$), T1R3 ($p<0.0001$), Gα-14 ($p<0.01$), and TRPM5 ($p<0.05$) compared to mCC females. The mCC males had more T1R3 expression than mCC females ($p<0.0001$). The mHH females had showed more Gα-14 expression than the mCC males ($p<0.01$).

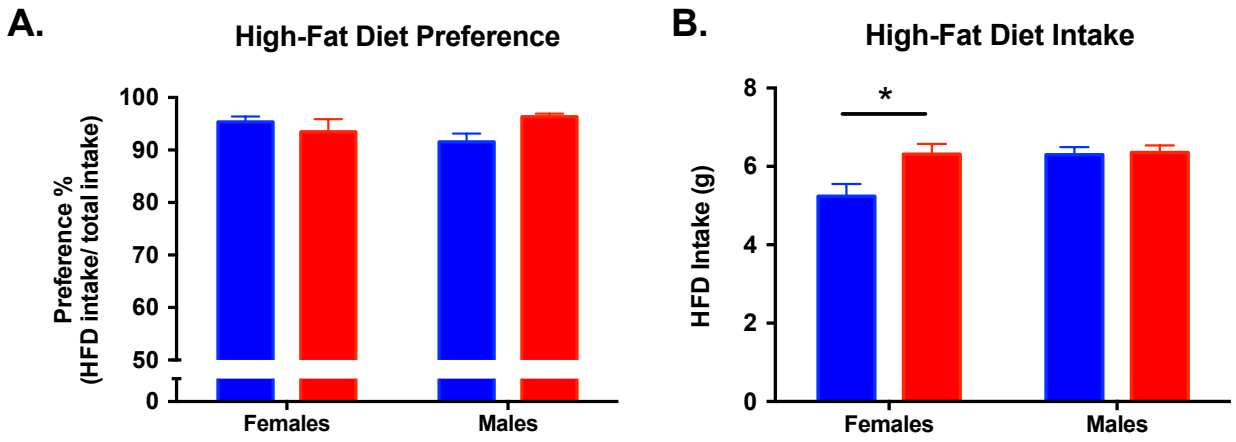


Figure 7. Diet preference and intake for NC vs HFD by (A) adult females (mCC n=8, mHH n=7) and (B) male offspring (n=9 per treatment). Values are expressed as means ± SEM. Data were analyzed by two-way ANOVA and post-hoc Tukey multiple comparisons (when maternal diet x sex interaction was identified). Significance shown here is based on comparisons between the maternal treatments within each sex; *p < 0.05. Blue = offspring of dams fed NC; Red = offspring of dams fed HFD.

Long-term effect of maternal HFD on the expression of umami, fat, and bitter taste receptors in the taste buds of the adult offspring

The results of the target were normalized to the housekeeping gene β-actin and relative values of target genes were calculated in relation to the mCC female group. Two-way ANOVA showed a main effect of maternal treatment on gene expression of T1R1 ($p<0.0001$) and T2R8 ($p=0.0054$). A significant interaction between maternal

treatment and sex was detected for CD36 (p=0.0095). Specifically, there was a main effect of sex on the expression of CD36 (p=0.0368) and GPR120 (p=0.0331), the putative taste receptors for fat taste. Tukey analysis indicated a significant increase in T1R1 (p<0.01) and T2R8 (p<0.05) in mHH females compared to mCC females (Figure 8C). The mHH males showed increased expression for CD36 (p<0.01) and decreased expression for GPR120 (p<0.05) when compared with mHH females. Recent work suggests that receptor protein expression in taste buds can accurately predicted by receptor mRNA expression (Lipchock et al 2013).

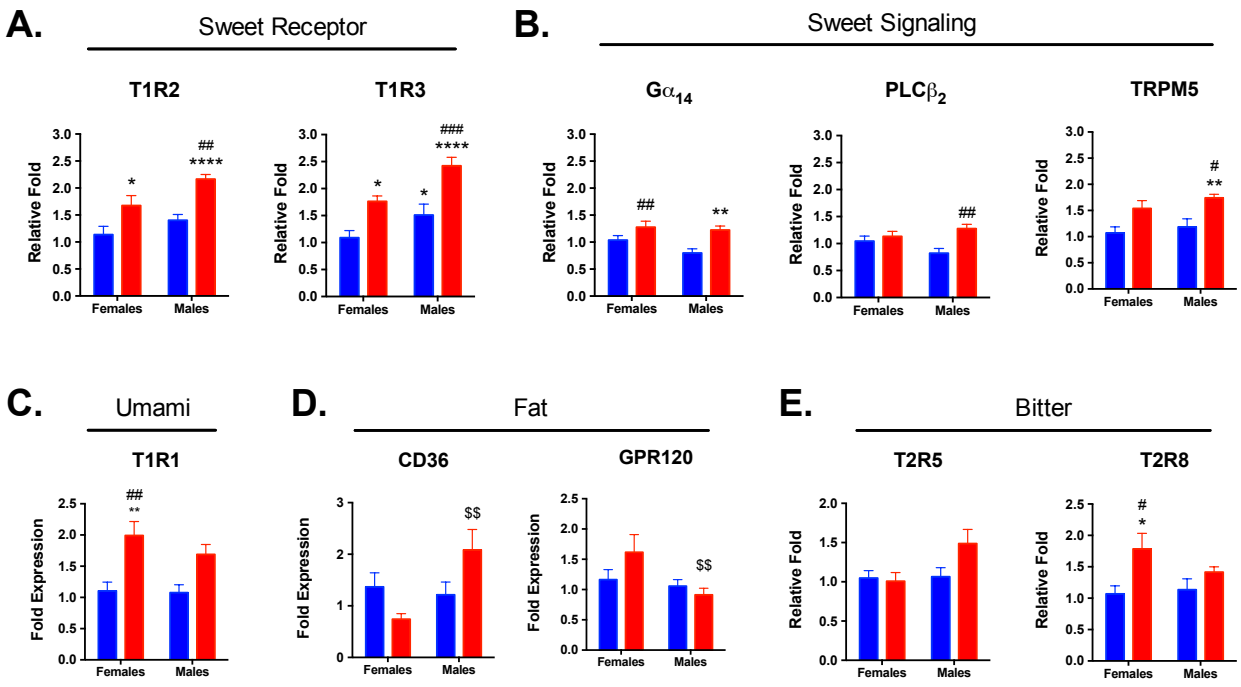


Figure 8. mRNA expression of sweet receptor subunits, sweet signaling components, and other taste receptors. Each biological sample (n=4 per group) was run in triplicate. Values are expressed as means ± SEM. Data were analyzed by two-way ANOVA and post-hoc Tukey multiple comparisons. Tukey post-hoc test: */**/***/**** p<0.05/0.01/0.001/0.0001 vs mCC females; #/## p<0.05, 0.01 vs mCC males; $$ p<0.01 vs mHH females. Blue = offspring of dams fed NC; Red = offspring of dams fed HFD.

Adult offspring have comparable taste bud morphology between treatment groups

To investigate how maternal obesity affects tongue morphology, we first determined whether the treatment caused any alterations to the fungiform papillae density on the surface of the tongue anterior (Figure 9). Increased fungiform density has been correlated with increased taste sensitivity at least in humans (Miller and Reedy 1990). Fungiform density was counted on the anterior tip of the tongues of mice from each treatment group as a measure of taste sensitivity, and there was no difference in fungiform density between the treatment groups.

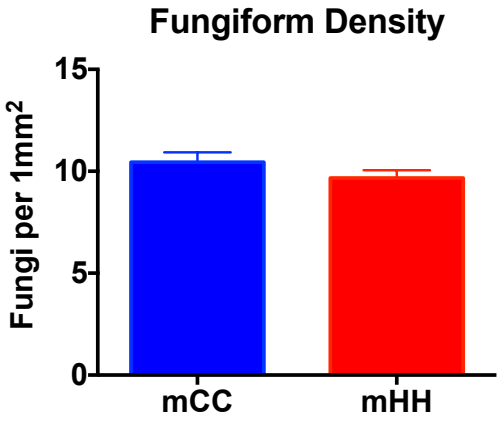


Figure 9. Fungiform density within 1mm x 1 mm square in mCC n=18 and mHH n=21, of both sexes combined. Student's t-test reveals no significant difference between treatment groups for fungiform density (p=0.2130). Blue = offspring of dams fed NC; Red = offspring of dams fed HFD.

Taste bud size was measured from ten taste buds from each mouse (n=8 for each maternal treatment). Analyses of H&E stained taste tissue reveal that taste bud size is also comparable between maternal treatment groups (Figure 10).

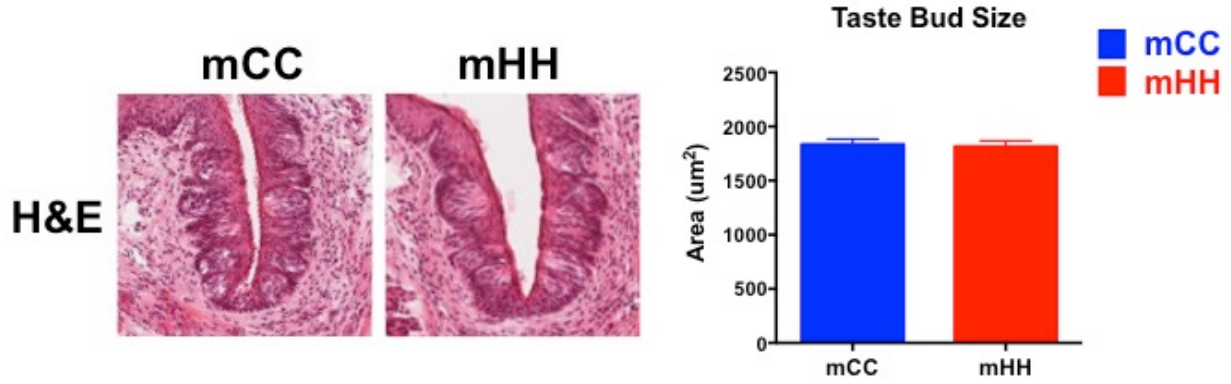


Figure 10. Taste bud size between treatment groups. Student's t-test reveals no significant difference between groups (p=0.7888).

We hypothesized that the mHH group showed greater sweet sensitivity because they had more taste buds in the CV and/or more sweet responding cells compared to the controls. Taste buds and taste cells were analyzed from immunofluorescent images using Poisson loglinear model of maternal diet (n=8, Figure 11). Analyses revealed no significant differences in taste bud numbers, sweet cells, and Type II taste cells (which includes sweet, umami, and bitter cells) between treatment groups (Table 11).

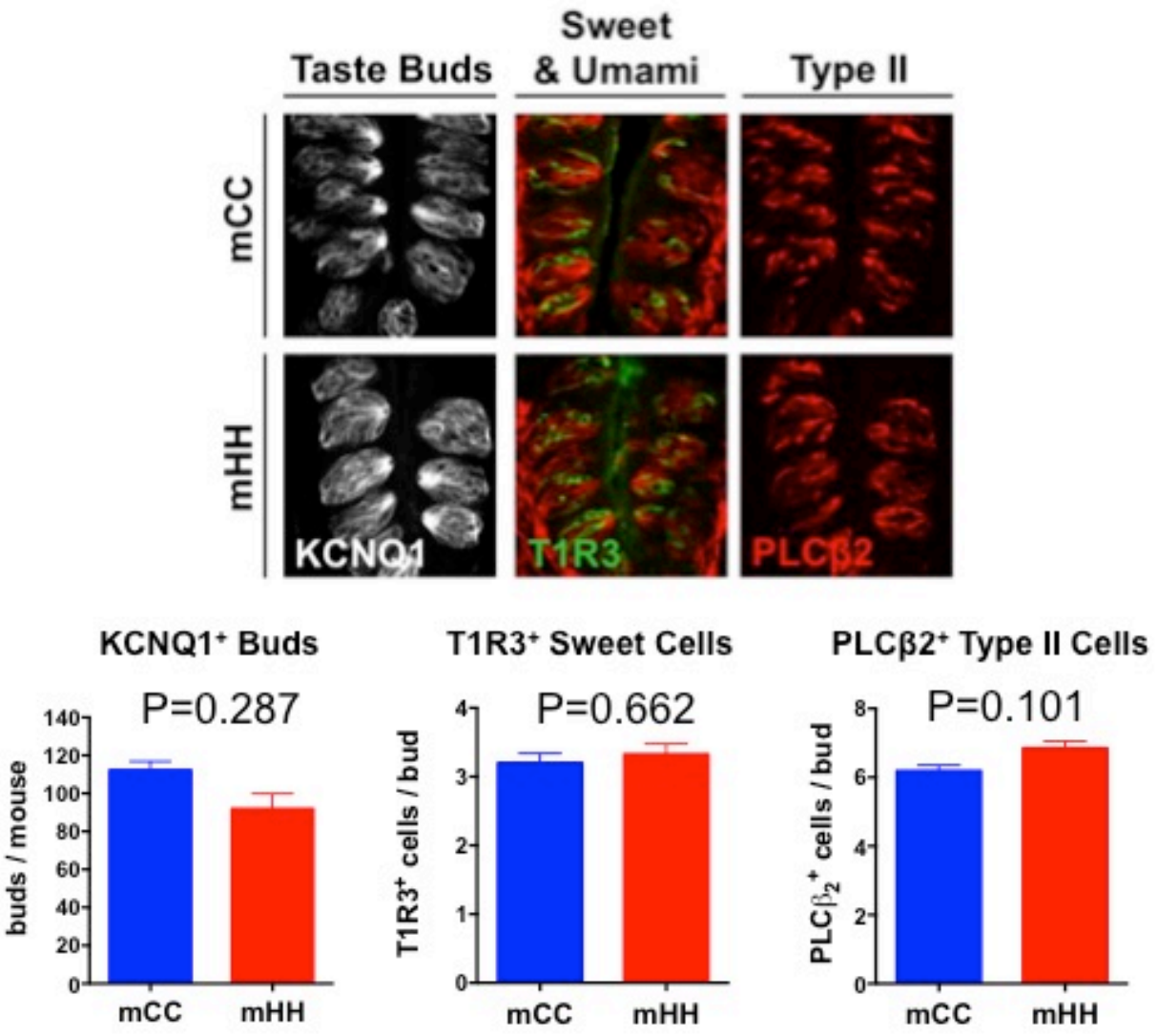


Figure 11. Number of taste buds in the circumvallate papillae, sweet cells, and Type II taste cells. The number of taste buds and cells were counted from every 8th section of immunofluorescent stains for KCNQ1, T1R3, and PLCβ2. Taste bud numbers were analyzed using Poisson loglinear model of maternal diet (n=8), significant level $p<0.05$.

Discussion

We carried out additional experiments in order to distinguish whether the effects we see on taste are due to maternal obesity prior to pregnancy (whether it be hormonal, inflammation, insulin resistance, hyperglycemia, etc.) or the dietary lipids that the offspring are exposed to in utero and through lactation. The HFD treatment before conception or solely during gestation/lactation did not elicit a change in sweet taste response in the offspring (Supplement Figure 3). It is possible heightened sweet responses could have been present early on due to early preexposure during lactation (Diaz-Cenzano and Chotro 2009), but the brief maternal HFD exposure was not enough to elicit a long-term change. Differential outcomes for males and females suggest that different processes underlie sex differences in palatable food consumption following maternal HFD exposure. Overall, this study is the first to demonstrate that maternal exposure to HFD during the perinatal period produced long-term consequences in taste behavior, feeding behavior, and gene expression of key sweet signaling components in a sex-dependent manner.

Effects of maternal HFD on body weight, blood glucose, baseline water and chow intake, fat pads, and hepatic lipids

In our study, the mHH adult males had a significantly lower body weight than controls at 8 weeks of age (Figure 3A); however the body weight between the two groups was no longer significantly different at 9 weeks of age during the behavior testing (Supplement Figure 1), which could be due to delayed physical maturation (Mendes-da-Silva et al 2014). The adult females showed no significant difference in between treatment groups, which corresponds with another study that showed maternal

HFD feeding for 3 months prior to mating had no impact of offspring body weight (Vucetic et al 2010). Additionally, there were no differences in perigonadal fat pad weights, blood glucose levels, and baseline water and chow intake between treatments for each sex (Figure 3B-E). These findings suggest that the adult offspring in our studies are similar at least in higher levels of physiological function. Some studies have shown contrasting results in which offspring of maternal HFD had increased epididymal fat pad weight and increased fatty liver in Swiss dams (Ashino et al 2012) and increased epididymal fat pads and liver mass in C3H/HeJ dams (Walter 2014).

Effects of maternal HFD on brief-access sweet response

The adult mHH female offspring show an enhanced licking response to sucrose compared to controls, while there was no change for the males between treatments. For the mHH females, the EC50 values were lower and licking responses were greater at the midrange sucrose concentrations. This indicates that it takes a lower concentration of sucrose to induce the same level of licking response as control females. We also examined adult offspring of dams fed HFD solely during the preconception period of 5 weeks prior to mating or solely during the gestation and lactation periods to determine if either time periods could elicit the same increase in sweet response; however, the adult offspring showed no significant difference in these treatment groups (Supplement figure 3). This is in line with similar studies by Treesukosol et al (2014) in rats. They showed no difference in sucrose concentration-dependent licking response in Lickometer testing on adult offspring when dams were fed HFD starting on day 2 of gestation. Our data implies that the females show an enhanced response to sweet taste, suggesting heightened sweet taste reactivity. Studies into 7-14 year old children and their sucrose

detection thresholds reveal that the more centrally obese children were more sensitive to sucrose relative to the other children examined (Joseph et al 2016).

Effects of maternal HFD on sucrose and sucralose preference and intake over 48 hours

Maternal HFD treatment resulted in increased sucrose and sucralose intake in the both offspring sexes compared to controls. Without any palatable stimuli reinforcement after weaning, both the female and male adult offspring drink more sucrose and sucralose than the controls. We found no difference in sucrose or sucralose preference between the treatment groups. In the mHH females, the enhanced sweet taste reactivity demonstrated with brief-access Lickometer testing may drive the increase in sucrose and sucralose intake. Although mHH males do not show different sweet brief-access responses compared to controls, they also consume more sucrose and sucralose.

High-fat diet preference and intake over 48 hours in the adult offspring

Although both sexes had never been directly exposed to HFD, the offspring of HFD fed dams consume more HFD than controls ($p<0.05$) suggesting that the enhanced sweet taste may contribute to the propensity for these offspring to over consume palatable foods. Thus, putting them at an increased risk for developing obesity. Our finding indicated that female but not male offspring of HFD dams consume more HFD than controls. These findings are identical with rat studies of maternal junk food feeding studies and offspring observed at weaning (Gugusheff et al 2013) and maternal low-protein diets where the adult females show increased fat intake compared to controls while the males did not (Bellinger et al 2004). This provides evidence

suggesting that the regulation of fat intake in females may be more susceptible to maternal HFD feeding than the males. However, our findings stand in contrast to work that has reported higher fat preference (Chang at al 2008, Vucetic et al 2010, Naef et al 2011, Ong and Muhlhausler 2011, Teegarden et al 2009) or higher HFD intake (Bayol et al 2007, Ong and Muhlhausler 2014, Tamashiro et al 2009, Teegarden et al 2009, Walker et al 2008, Treesukosol et al 2014) in offspring of maternal HFD treatment. Differences in methodology, such as the amount of fat in the treatment diets and timing of maternal HFD treatment and mating, may account for these differences in findings. The differences in fat intake between the sexes may be due to differences in maturation and physiological developmental rates, however further investigation is needed. A possible explanation for this sex polymorphism could be due to hormonal differences such as with estrogen levels. Differences in gonadal hormones between the sexes, such as estradiol, have been implicated in the regulation of feeding behavior and intake (Eckel 2011). Hypothetically, estrogen could influence factors that determine taste receptor cell fate (Barlow 2015), which could ultimately impact the type of cells that are added to the taste bud.

Maternal HFD effects on gene expression in the taste buds of the adult offspring

Sweet taste usually predicts caloric properties of food and is known to induce a hedonic response to promote ingestion (Anderson 1995). We examined gene expression levels for the sweet receptors subunits T1R2 and T1R3 as well as several intracellular signaling elements, including Gα-gustducin, PLCβ2 (phospholipase C- β2), and TRPM5 (a cation channel member of the transient receptor potential superfamily, subfamily M, member 5). These elements act downstream of the sweet receptor and

play a role in the sweet signaling process. Sweet receptor and these downstream signaling elements have been shown to be modulated in some physiology systems leading to altered feeding behavior. For example, chronic stress decreases T1R3 expression (Okamoto et al 2010, Parker et al 2014) and mRNA expression of Gα-gustducin has been found to be increased in the taste buds of diabetic rats (Zhou et al 2008).

In the mHH adult females we found increased T1R2 and T1R3 sweet receptor expression and increased TRMP5 levels, a component of the sweet signaling pathway (Figure 8). This was associated with increased intake for HFD, sucrose, and sucralose. Although the functional consequences of increased sweet receptor expression and TRPM5 are unknown, KO mouse studies of TRPM5 and T1R3 demonstrate reduced overeating of high-caloric carbohydrates and fats (Glendinning et al 2012, Larsson et al 2015) and loss of sweet, umami, fat, and bitter taste responses (Zhang et al 2003, Damak et al 2006, Liu et al 2011). The increase in T1R expression in the adult offspring of maternal HFD is counterintuitive considering studies in rodent models of diet induced obesity using HFD (i.e. direct HFD feeding during adulthood and not via maternal exposure). Rats fed HFD for 6 weeks showed decreased T1R3 expression, which was associated with lower intake and preference ratios for saccharin solutions (Chen et al 2010).

Of the genes investigated in the adult offspring taste buds, T1R1 and T2R8 were uniquely unregulated in the mHH females over all the other groups (i.e. males of both treatments and the control females). The increase in T1R1 expression was unexpected as rodent studies into maternal HFD effects on gut tissue on the weanlings found

increase T1R1 expression (Reynolds et al 2015) and effects on cardiac ventricle tissue found T1R1 to be unchanged (Raipuria et al 2015). Our study indicates an increase in T1R1 expression in the taste buds of adult offspring of maternal HFD feeding. The differences in whether T1R1 is decrease, increased, or unchanged in these studies could be due to the route of maternal exposure. The taste system is exposed to the outside environment, especially while in utero or during the process of suckling. This suggests that the taste cells and their receptors are potentially being stimulated at a higher level than other organs such as the gut and cardiac ventricles that become inward facing during later development.

The mHH male offspring in this study had higher levels of CD36 gene expression in their taste buds than the mC male controls. The mHH females showed a trend towards decreased CD36 expression, although not significant. CD36 is a putative fat taste receptor. Other studies have shown maternal HFD decreased CD36 expression in skeletal muscle in male offspring (study did not investigate female offspring; Walter and Klaus 2014), increased CD36 expression in the livers of the male offspring (study did not investigate female offspring; Zheng et al 2014), and resulted in no change in CD36 expression the guts of offspring from NC and HFD fed dams (Reynolds et al 2015).

Maternal Obesity and Epigenetic Programming

This work provides evidence that the obese maternal environment produces long-term programming effects on the offspring taste system. Our results demonstrate that offspring of maternal obesity weaned onto NC show increases in T1R mRNA expression into adulthood past 8 weeks age. Permanent alterations and stable long-term repression of some genes maintained through cell division, as suggested by our

results, can be linked to DNA methylation and histone modification (Cedar and Bergman 2009). A maternal HFD has been shown to affect epigenetic machinery (Panchenko et al 2016). The increased preference for high-fat and high-sucrose in adult offspring of maternal HFD is associated with global and gene specific decreases in DNA methylation in the offspring brain at 20 weeks age (Vucetic et al 2010) that persists to 40-50 weeks of age. In rodent studies, reversal of DNA hypomethylation through supplementation of the maternal diet with methyl donors can ameliorate increases in body weight gain in the offspring of the HFD dams (Carlin et al 2013, Cordero et al 2013) and transgenerational amplification of obesity (Waterland et al 2008). Interestingly, methyl donor supplementation also reduces the increased fat preference in adult offspring of HFD dams; although, the attenuation is significant in the males, but not as pronounced in the females (Carlin et al 2013). This may be due to males showing a significantly stronger preference for fat (Day et al 2012). Thus, alterations to the epigenome in early ontogenesis could be a mechanism by which gene expression patterns are passed from the mother to the offspring resulting in persistent phenotypes into adulthood.

Maternal nutrient restriction or low-protein diet can also program a preference for HFD in the offspring

In our experiments, we fed the dams with HFD, which means that they are potentially consuming less protein because it was replaced by lard. This is important to note because maternal malnutrition or low-protein diets throughout the prenatal period have also been shown to program an increased preference for high-fat foods and adult obesity. Thus, the overall maternal nutrient restriction or low-protein could contribute to

that results we see. Adult 12-week old rats of pregnant rats fed low-protein diets throughout gestation consume more fat, less carbohydrates, and similar amounts of protein when compared to their lean counterparts (Bellinger et al 2004). Similar to our experiments, the body weight was comparable between both treatment groups at 12 weeks of age. Suggesting that early life exposure to maternal undernutrition programs the offspring to have a preference for high-fat foods.

Parental/ Maternal Diet Alters Taste Receptor Expression

Although taste receptors were first described in taste cells in response to nutrients in the environment, taste receptors have since been discovered and described throughout the body (Finger and Kinnamon 2011, Li 2013). In other organ systems, instead of being used for tasting food, these taste receptors instead act as nutrient sensors. For example, instead of tasting sweetness in food, sweet sensing receptors of the pancreas (with the same exact protein structure of that found in taste cells, although signaling mechanisms may vary) sense glucose in the environment and in response trigger insulin release (Nakagawa et al 2009). Thus, it is interesting to consider that the maternal HFD may be stimulating nutrient receptors in the mother and/or offspring during development leading to alterations to the taste epigenome, which could be detrimental to the offspring (i.e. propensity towards an obesogenic phenotype) or beneficial through adaptive programming. Other hypotheses include direct modulation of epigenetic machinery through the diet or affects from secondary factors linked with maternal obesity such as elevated maternal circulating leptin, insulin, glucose, and inflammatory cytokines.

Some rodent studies demonstrate that taste receptor mRNA expression, at least in the gut, heart, and brain, can be altered by the maternal diet. In a rodent study of gut tissue from male and female offspring at weaning day 24 of dams fed HFD during gestation and lactation, expression of Tas1r1 in both sexes was increased in the offspring of HFD dams, while Tas1r3 was also reduced in the males, but not the females (Reynolds et al 2015). Interestingly, maternal diet supplementation with an anti-inflammatory lipid, conjugated linoleic acid (CLA), was able to increase Tas1R3 expression in the male offspring, essentially reversing the effects seen with maternal HFD (Reynolds et al 2015). In cardiac studies of 19-day-old Sprague-Dawley rat pups, mRNA expression of bitter receptors Tas2r126 and Tas2r143 in cardiac ventricle tissue was lower, while the umami receptor subunits Tas1r1 and Tas1r3 were unchanged, in the pups of obese dams compared to their lean counterparts (Raipuria et al 2015). Beyond this study, there remains a dearth of published research into the effect of diet on bitter taste receptor expression. A maternal low-fat diet in a psammonys obesus (Israeli sand rat) model lead to offspring having decreased Tas1r1 mRNA expression in hypothalamic tissue, which was associated with increased methylation on the Tas1r1 gene as revealed by methyl-CpG binding domain capture and deep sequencing (Khurana et al 2016). Studies using in vitro fertilization show that epigenetic factors in gametes play an important role in the transmission of parental obesity to the next generation (Huypens et al 2016), and mother-child associations for obesity have been shown to be significantly greater than father-child associations (Whitaker et al 2010). Thus, maternal obesity may mediate long-term changes in gene expression, particularly expression of taste receptors, through epigenetic regulation such as changes in DNA

methylation or histone acetylation. See supplement for further discussion of taste receptor expression regulation by epigenetic mechanisms.

Conclusions

Adult females of HFD fed dams show increased sweet receptor mRNA expression, increased licking responses to sucrose, and over consume sucrose, sucralose, and high-fat diet. The results here suggest taste bud function can be altered through maternal obesity and add to our understanding of how maternal obesity contributes to the offspring's risk of developing obesity.

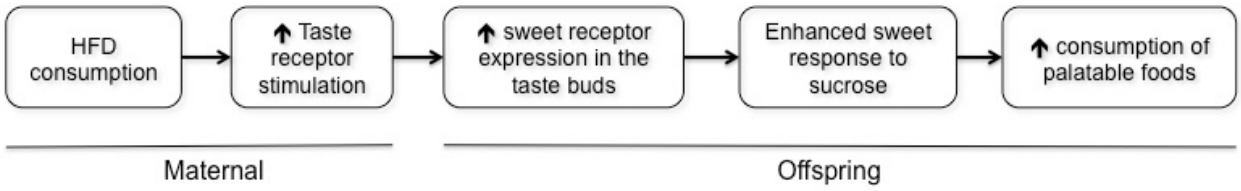


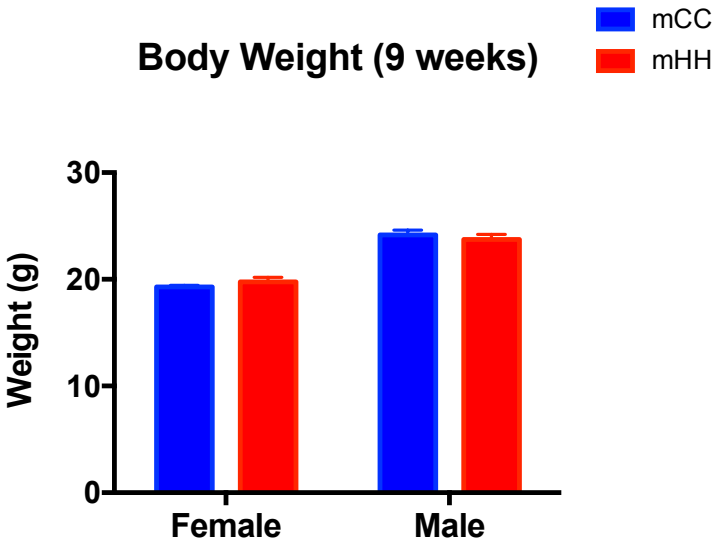
Figure 12. Summary of proposed mechanism through which the maternal HFD regulates sweet taste expression at the level of the taste buds resulting in a preference for palatable foods in the offspring. We speculate that the expression of sweet signaling related genes may be promoted through long-term maternal stimulation to palatable HFD. The exact mechanism by which this occurs requires further investigation.

Collectively, the current study could not give a definite answer about the exact mechanisms of how maternal HF treatment changes sweet responses and gene expression in the adult female offspring. The current results from sucrose and HFD preference testing, although identical in findings with some studies (Gugusheff et al 2013, Bellinger et al 2004), stand in contrast with previous findings (Chang at al 2008, Vucetic et al 2010, Naef et al 2011, Ong and Muhlhausler 2011, Teegarden et al 2009). As discussed above, our findings may be be a reflection of differences in methodology

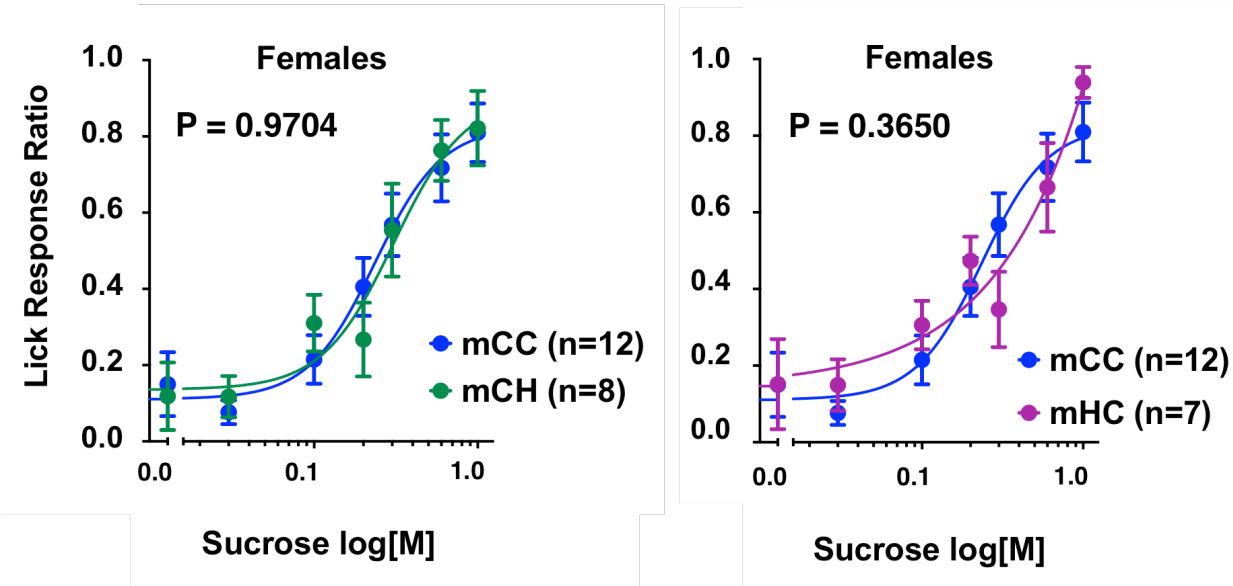
(i.e. different HFD composition, timing of maternal treatments, age at which offspring were examined). We encourage future investigations to examine offspring taste behavior over the course of development or during advanced age (i.e. 6 months) as opposed to starting at 8 weeks as was done here. Additionally, a post-natal HFD (i.e. for 3-5 weeks after weaning) and the long-term impact on the taste system in the developing offspring or at adulthood would be interesting as secondary data analysis of pregnant women suggest that the post-natal diet of fruit and vegetables was correlated with childhood diet and acceptability (Ashman et al 2014). Further studies could investigate possible epigenetic regulation of the taste receptors in the adult offspring, by supplementing the maternal diet with methyl donors to reverse the hypothesized DNA hypomethylation of taste receptors in the adult offspring. The long-term effects of maternal HFD feeding on the adult offspring can be further investigated by studying the taste system in the F2 to determine if the phenotype has transgenerational persistance suggesting evidence of long-term maternal programming. A limitation in all maternal overnutrition studies such as this is whether the long-term effects on the offspring taste system are a direct result of the increased dietary fat, a reduction in protein and/or carbohydrates as a result of the diet being higher in fat content, or through differences in micronutrient composition (Chalvon-Demersay et al 2017). Future studies could look into the role of the microbiome, more specifically, whether a maternal supplementation of prebiotics could ameliorate the enhanced sweet response we see in the adult offspring females. Prebiotic intake during pregnancy and lactation has been shown to improve maternal metabolism and attenuate some nutritional programming of the offspring due to maternal HFD exposure (Paul et al 2016). Further investigation is

required to investigate the exact mechanisms by which maternal obesity leads to an increase in sweet receptor expression in the offspring and results in the adult female offspring showing a significant increase in brief-access licking response to sucrose when the males did not. Regardless of the root of how maternal obesity increases the offspring's risk for developing obesity later in life, it is clear that as assessed in brief access tests, sweet responses are not universally increased by maternal HFD during the perinatal period for both sexes. From a public health standpoint, improving our knowledge of the prenatal and early postnatal factors that program subsequent obesity in the offspring may provide insight into therapeutic targets for fighting the obesity epidemic, a disease that is easier to prevent than to cure.

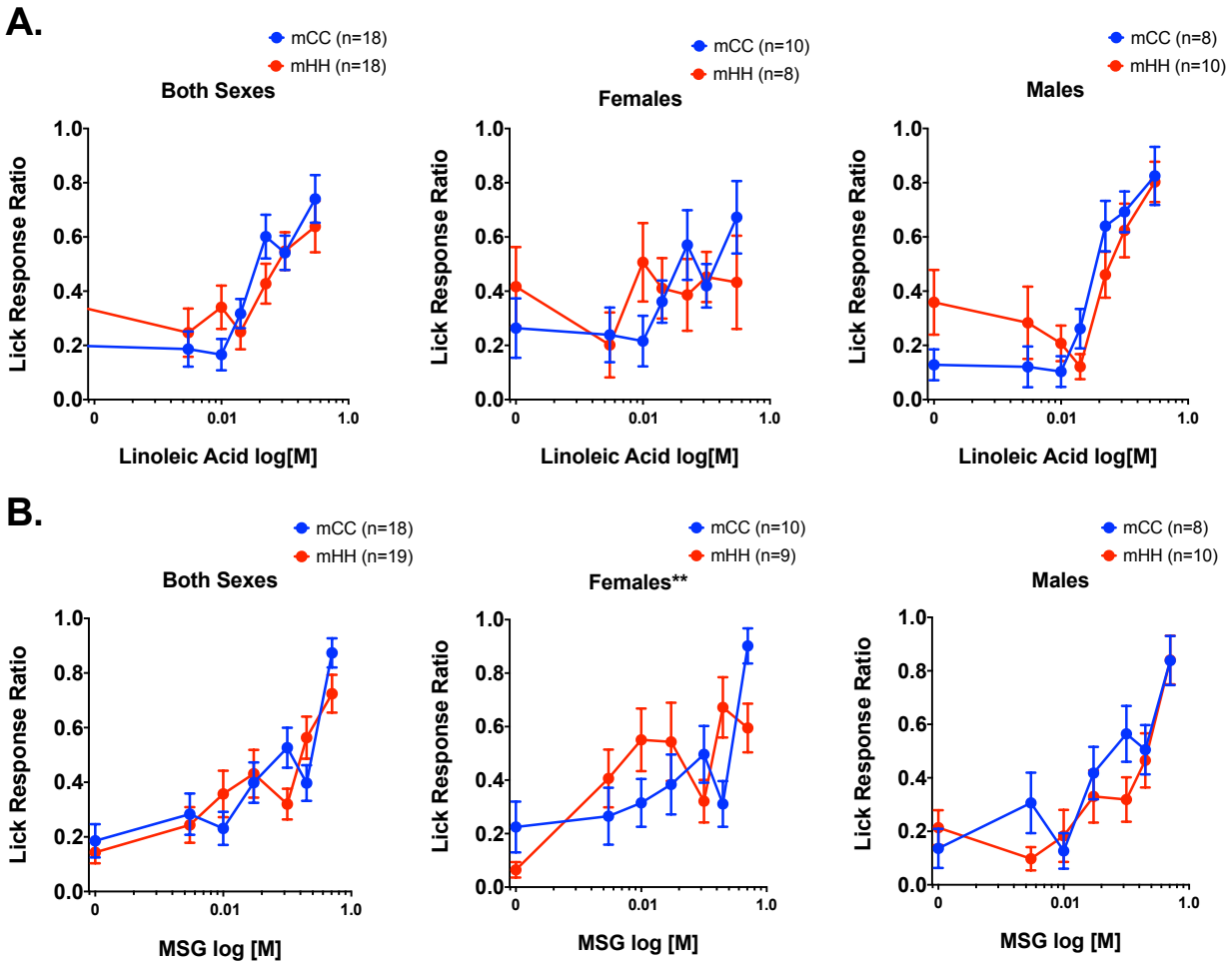
Supplemental Figures



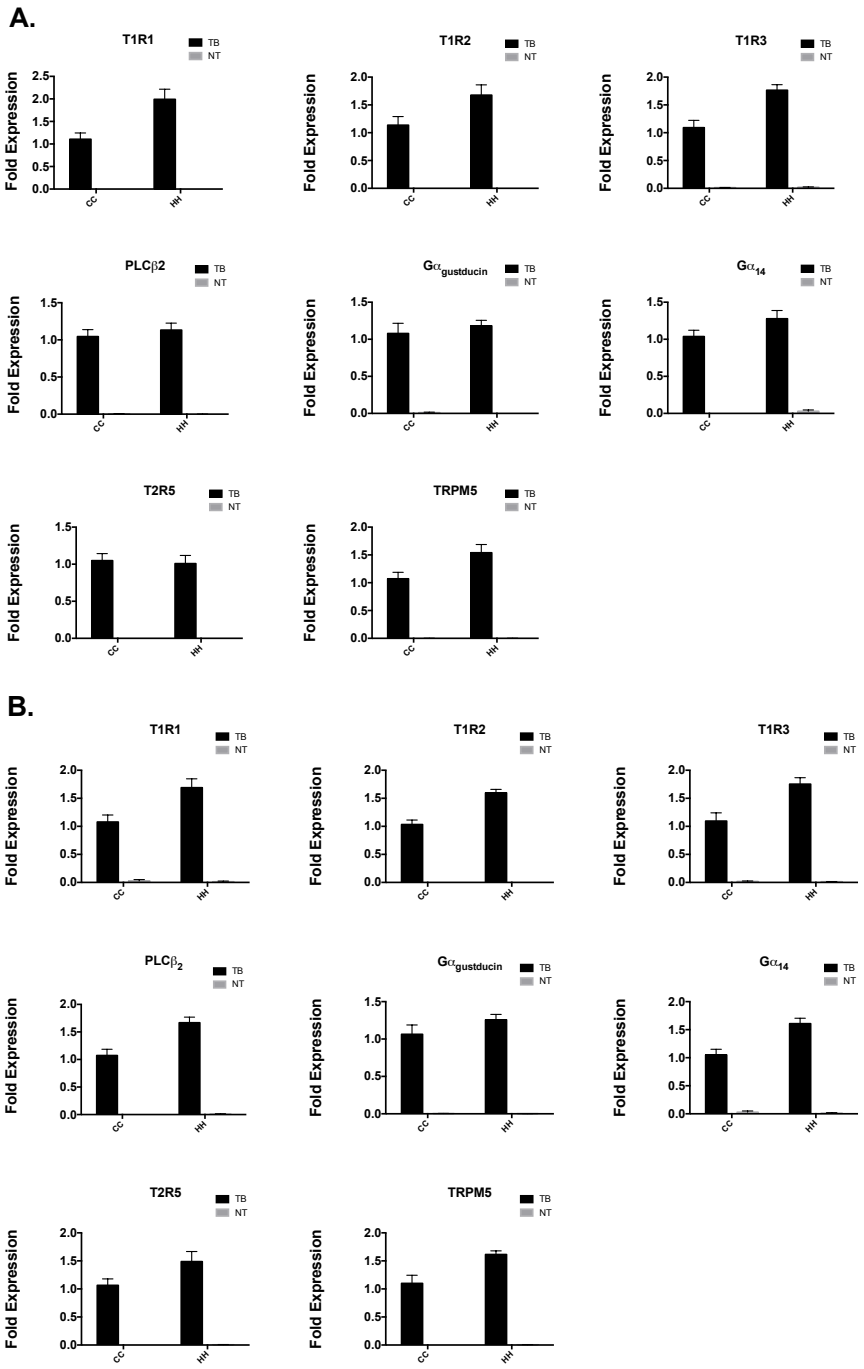
Supplement Figure 1. At 9 weeks of age the body weights for both sexes between the maternal treatments is comparable. Two-way ANOVA indicate no significant interaction between maternal treatment and sex ($F_{1,35}$=1.313, p=0.2596) with significant level p<0.05.



Supplement Figure 2. The intermediate group mCH represents excess fat consumption only during gestation/ lactation (i.e. gestational weight gain). The mHC group represents high-fat consumption only prior to conception. In the females, maternal treatment of HFD before nor after conception was enough to change sweet taste responses in the adult female. Similarly, no change in sweet response was found in the males (data not shown) due to these intermediate maternal treatments.



Supplement Figure 3. Lickometer responses to linoleic acid (tastant for fat tested at 0, 0.003, 0.01, 0.02, 0.05, 0.1, and 0.3 M) and mono-sodium glutamate (tastant for umami tested at 0, 0.003, 0.01, 0.02, 0.05, 0.1, and 0.3 M) were carried out on mCC and mHH mice of both sexes. Nonlinear regression sigmoidal dose-response curve analysis could not be carried out, so instead data was analyzing using two-way ANOVA. When interaction between maternal treatment tastant concentration was significant, $p>0.05$ then post-hoc Sidak multiple comparisons for between treatment groups was performed. The females had a significant interaction treatment x MSG conc (**$p = 0.0068$); however, there were no significant differences between the treatment groups at any MSG concentration ($p = 0.4950$).



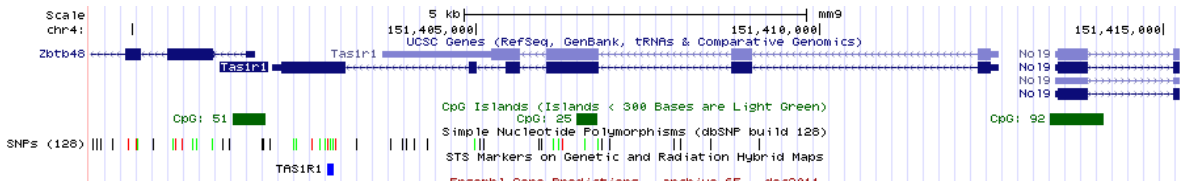
Supplement Figure 4. Non-taste samples were processed and analyzed in parallel as a negative control for taste samples in females (A) and males (B). All genes examined were taste specific, so we saw little to no expression in all non-taste samples.

Epigenetic Regulation of Taste Receptor Expression (Supplemental Discussion)

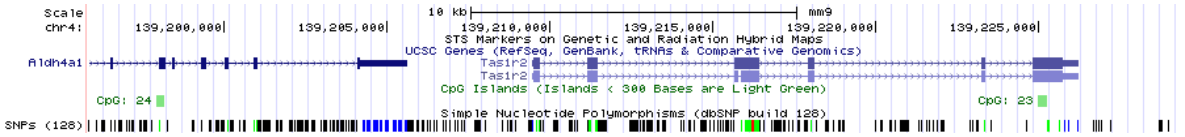
To begin investigating whether epigenetic regulation may be involved with the changes in Tas1R expression we see, we searched the gene sequences for possible sites of DNA methylation such as CpG islands, particularly in the promoter regions. DNA methylation occurs at CpG sites to form 5-methylcytosines at the 5' position of the pyrimidine ring of the cytosine residue. Methylation of multiple CpG sites in CpG islands within promoters is associated with stable silencing of genes (Bird 2002). Using the UCSC Genome Browser on Mouse July 2007 (NCBI37/mm9), we searched for CpG islands on Tas1r1, Tas1r2, and Tas1r3. Tas1r1 has one CpG island in the middle of the gene on the third exon, Tas1r2 has a CpG island at the end of the gene on the last exon, and Tas1R3 has no CpG islands. Taken together, the Tas1R genes do not have typical CpG island sites that would suggest regulation by DNA methylation.

Tas1r1 does not have CpG islands in the promoter region as one might hope to find, although there is one study that demonstrates Tas1r1 hypermethylation is associated with decreased mRNA expression. Khurana et al (2016) studied Psammonys obesus (Israeli sand rat) offspring exposed to a maternal low-fat parental diet *in utero*. These offspring, when compared to controls, were heavier and had increased circulating insulin and glucose levels. Additionally, methyl-CpG binding domain capture and deep sequencing (MBD-seq) reveal increased methylation of Tas1r1 in hypothalamic tissue, which was associated with decreased mRNA expression analyzed using qRT-PCR. Their pathway analysis revealed novel DNA methylation of hypothalamic genes associated with neurological function, nutrient sensing, appetite, and energy balance (Khurana et al 2016).

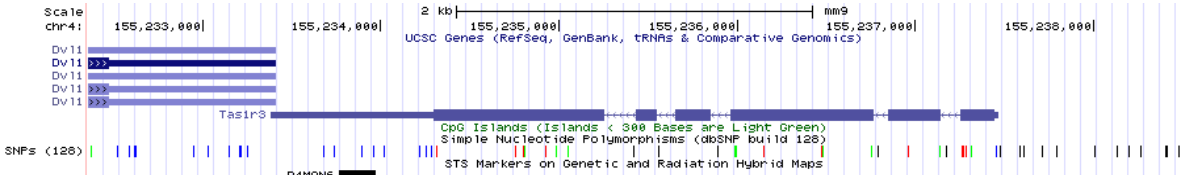
Tas1r1 (taste receptor type 1 member 1 precursor):



Tas1r2 (taste receptor type 1 member 2 precursor):



Tas1r3 (taste receptor type 1 member 3 precursor):



Supplement Figure 5. UCSC Genome Browser tracks for Tas1Rs and their CpG islands.

This suggests that in our experiments, the maternal HFD exposure may have the opposite effect as we see increased expression of Tas1r1 in the taste buds using qRT-PCR, which could be associated with hypomethylation of the Tas1R1 gene.

Although Tas1R3 does not have any CpG islands, there is a strong association between single-nucleotide polymorphisms (SNPs) located in Tas1R3 at positions 21572 (rs307355) and 21266 (rs35744813), with human taste sensitivity to sucrose (Fushan et al 2009). Although the Tas1r3 promoter in humans and mice is in an evolutionary conserved region, the distal promoter region of Tas1R3 contains a composite cis-acting element that strongly silences promoter activity, which provides an attractive candidate site of possible epigenetic regulation in our mouse studies. Unfortunately, the DNA sequence of this distal promoter region in higher mammals (humans, chimp, orangutan)

shares no significant similarities with mouse, rat, horse, or dog. Overall, direct modulation of the Tas1rs through epigenetic regulation appears to be more complex than simple hypo or hypermethylation of CpG islands in promoter regions.

Regulation of Taste Receptor Expression not related to methylation

Originally, Tas1r3 was discovered in gustatory tissue, but now it is known to be expressed in intestine, pancreatic beta-cells, skeletal muscle, and heart. Kokabu et al (2015) characterized the genomic region upstream of the annotated transcriptional start of human Tas1r3 that contains the repressive element, which was missing in mouse/rats similar to the findings of Fushan et al (2009). Using cell culture, they found muscle regulatory factors MyoD and Myogenin regulate Tas1r3 expression. As skeletal myogenesis progressed in murine myoblast C2C12 cells, the expression of Tas1r3 and Tas1r1 increased (Kokabu et al 2015). Although it is unknown whether muscle regulatory factors are expressed in taste, similar modulatory mechanisms may also exist for taste receptors at the level of the taste buds.

Okamoto et al (2010) show that chronic stress to rats decreases T1R3 expression in fungiform papillae. Based on nerve recording of the chorda tympani, sweet and umami responses were decreased while the three other basic tastes did not change. Thus it appears that stress can induce inhibition of Tas1r3 expression. Interestingly, Parker et al (2014) found that glucocorticoid receptors are co-localized with most Tas1r3+ mouse taste cells. When mice are restraint stressed the GR mobilizes to the nucleus of T1R3 taste cells. Thus, translocation of GR to the nucleus of taste cells is inducible by behavioral stress and could be a possible mechanism for activation of particular genes in taste receptor cells by GR action. Additionally, Ogawa

et al (2015) found that adrenalectomized (ADX) rats had fewer fungiform papillae and lower Tas1r3 mRNA expression in taste than ADX-shams, suggesting that low levels of glucocorticoid is necessary for Tas1r3 induction. When administered with a small dose of dexamethasone (DEX, which mimics glucocorticoid) Tas1r3 expression was restored to the level of the ADX-shams; however, higher levels decreased/ inhibited Tas1r3 mRNA expression. Thus, taste receptor expression can be modulated by non-epigenetic means such as circulating glucocorticoid levels.

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CHAPTER 5

Maternal High-sucrose Diet at Levels Relevant to Human Consumption Does not Impact Offspring Taste Function

Abstract

The aims of this study were to test the effects of sucrose supplementation to pregnant mice on the taste system of their offspring, and to test if similar effects could be elicited using supplementation of the non-nutritive sweetener sucralose. We hypothesized that feeding a dam an intensely sweet solution would produce offspring that also prefer sweet taste when compared to offspring of dams only given water. In these studies, females were mated following a 4-week period in which one group was given sucrose or sucralose in addition to water *ad libitum* and the control group was given chow and water only. Sucrose and sucralose solutions were removed two week after parturition to prevent direct drinking by the offspring. The adult offspring at 8 weeks of age for both the sucrose and sucralose maternal supplementation groups show no change in sucrose and sucralose responses. No effect of maternal sweet supplementation was detected at the taste bud level (i.e. fungiform density and taste bud gene expression remained unchanged). Overall, this study suggests that sucrose and sucralose consumption at human-relevant levels during pregnancy and lactation do not produce any long-term effects on the adult offspring taste system.

Introduction

From an evolutionary perspective, there may be some advantage to taste being susceptible to fine tuning by the mother's diet during the perinatal period. Thus the offspring may innately be drawn to already familiar, "safe" foods. Given the evidence that gestational diabetes is associated with greater cravings and preferences for sweetened foods in pregnant mothers, it remains unknown how a diet heavy in sugars can affect sweet taste response in the offspring (Belzer et al 2010). Increased prevalence of obesity has been linked to increased dietary of fats, sugar, and reduced physical activity (Andersen 2000). Increased non-nutritive sweetener consumption is also associated with increased body weight, and a higher prevalence of obesity (Fowler et al 2008, Mattes and Popkin 2009, Yang 2010). Artificially sweetened beverage consumption during pregnancy is also linked to a higher body mass index in the offspring (Azad et al 2016).

Studies feeding 10% sucrose to rats at weaning age (21 days) for 3 weeks result in an increased sucrose preference relative to control rats receiving just water at weaning (Sato et al 1991). To our knowledge, a study of maternal sucrose treatment during the pregnancy and lactation period and its effects on the offspring's preference for sucrose has yet to be carried out. Although a number of studies have already looked at the impact of non-nutritive sweeteners during pregnancy and lactation and their impact on adult offspring metabolism and metabolic disease (Pepino 2015, Araújo et al 2014), a better understanding of how sweet taste is modulated through maternal sweet exposure may help us find new ways to modulate sweet taste in relation to dieting. This is particularly relevant considering that an overall reduction in sugar-sweetened beverage

consumption can reduce the prevalence of obesity and obesity-related diseases (Hu 2013).

Sweet taste modulation in offspring by maternal acesulfame-K exposure

Rodent studies by Zhang GH et al (2011) demonstrate that acesulfame-K, one of the most widely used non-nutritive sweeteners, is ingested prenatally through the mother's amniotic fluid, as well as postnatal through breast milk. This early acesulfame-K exposure is capable of increasing the offspring's preference for acesulfame-K by ~25%, as well as for sucrose by ~30% in two-bottle preference tests. Although follow-up studies focused on early intraoral acesulfame-K exposure in pups, instead of *in utero*, the same group was able to show changes in offspring taste buds for regulators and components of sweet signal transduction such as T1R2, leptin (OB-Rb) and endocannabinoid (CB1) receptors (Li et al 2013). Interestingly, Gα-gustducin expression, believed to be a reliable marker for chemosensitive cells, was also increased in fungiform taste buds (Chen et al 2013). These findings suggest that maternal ingestive behavior can impact fetal taste programming. It should be noted that acesulfame-K maternal treatment was *ad libitum* in these studies, and the total intake amount equated to more than 20-fold of average daily intake levels (Mattes and Popkin 2009).

Sucralose – a non-nutritive sweetener

Like acesulfame-K, sucralose can be detected in breast milk (Sylvetsky et al 2015). The perception of sweet taste by both sugars and artificial sweeteners is peripherally mediated by T1R3 and T1R2 heterodimers on the tongue (Chandrashekar et al 2006). Most artificial sweeteners bind to taste receptors with greater affinity than

sucrose (Nie et al 2005) and sucralose is about 600 times sweeter than sucrose (FDA 1998). Not only is sucralose sweet at low doses, but it is also excreted almost entirely unchanged, overall producing two minor metabolites as measured in mouse urine (John et al 2000), which contributes to its zero contribution to caloric intake and overall safety for consumption (Rodero et al 2009). Sucrose activates taste pathway regions in the brain more than sucralose, suggesting that sucrose and sucralose may result in varying physiological brain responses, despite it being difficult for participants to distinguish the difference in taste between sucrose and sucralose (Frank et al 2008). A preference for sucralose can predict behavioral responses to sweet and bittersweet tastants (Loney et al 2012) and has been correlated with less obvious phenomena such as drug seeking behavior, impulsivity, and risk taking behavior (Dess et al 1998, Perry et al 2007, Anker et al 2008, Carroll et al 2008).

The aims of this study were, first, to test the effects of sucrose supplementation in pregnant mice on the taste system of their offspring and second, to test if these same effects could be elicited using supplementation of the non-nutritive sweetener sucralose.

Methods

Animals

In-house bred virgin C57BL/6 female mice (n=3 each group) at 8 weeks of age were randomly assigned to receive water, a sucrose solution (0.623M, food grade), or a sucralose solution (6.7mM, food grade) daily for 4 weeks prior to mating and then continued to receive their respective supplements throughout the gestation/ lactation period until 2 weeks postpartum.

Sweet Maternal Treatment and Exposure

Sucrose and sucralose treatments were administered orally via liquid ration provided at the same time every day to mimic human consumption of a daily-sweetened beverage. Mice found the solutions appetitive and consumed the full amount. The recommended daily intake for sucrose in women is 100 calories (or 24 grams or 6 teaspoons of sugar). The FDA approved daily intake of sucralose is 5 mg/ kg BW. These daily recommendations were adjusted for typical daily consumption by multiplying by 3.5 times, i.e. NHANES data show that the average person consumes 83 grams of sugar, which is 3.5 times the recommended daily maximum (Marriott et al 2010). Then the dose for mice was extrapolated from the human equivalent dose (HED) using body surface area given Km values for mouse to be 3 and human 37 (assuming a human is 60 kg; Reagan-Shaw et al 2007). This yielded concentrations of 0.623M sucrose and 6.7 mM sucralose, both of which fall within appetitive ranges for mice.

$$HED\ \left(\frac{mg}{kg}\right) = \ Animal\ dose\ \left(\frac{mg}{kg}\right) \cdot \frac{Animal\ Km}{Human\ Km}$$

Sweet Taste Behavior Assay – Brief-Access Lickometer

Taste responses (i.e. taste-related affective potency of the stimuli) were measured using a brief-access Davis Lickometer, which minimizes confounding factors such as appetite and post-ingestive effects. This method quantifies immediate lick responses to extremely small volumes of sapid solutions. The training and testing schedule was adapted from Glendinning et al (2002) and Glendinning et al (2005). Testing was conducted under simulated dark cycle conditions (under red lights). For sweet

testing, a range of sucrose (0, 0.03, 0.1, 0.2, 0.3, 0.6, 1.0 M) and sucralose (0, 0.03, 0.1, 0.3, 1, 3, 10 mM) concentrations was used on separate testing days. Each test session lasted no more than one hour, during which the mouse could initiate up to 5 blocks of 7 concentrations (i.e. 35 total presentations).

All Lickometer data were downloaded as .csv files and imported into Excel for further data analysis. Lick responses were normalized, fit to nonlinear variable slope concentration-response curves, and compared using extra sum-of-squares F test. The number of licks for each concentration was averaged within each mouse. These averages were then divided by the maximal lick rate and subtracted from the minimum rate within each mouse yielding the standardized lick ratio. A lick ratio of 0.0 indicates that the sucrose concentration elicited minimal licking over water, whereas a value of 1.0 indicates maximal licking; thus, controlling for individual differences in local lick rate for each mouse. Tastant concentration–lick ratio response curves were fitted to the mean data for each group using a classical four parameter logistic sigmoidal dose–response equation in the nonlinear regression suite of GraphPad Prism (v5.0).

Two Bottle Testing – Sucrose and Sucralose

Adult offspring were single housed and provided normal chow (NC) ad libitum. Mice were trained to consume water from two bottles for 48 hours. Following this, mice were tested with water vs sucrose (0.3 M sucrose), and then with water vs sucralose (1.0 mM sucralose). Mice were given simultaneous access to the two bottles and consumption was measured over 48 hours. Starting bottle order was randomly assigned and sides were switched after 24 hours. Sucrose and sucralose preferences were

calculated as a percentage of sucrose intake divided by total fluid intake. For more details, see Appendix – SOP Two Bottle Testing.

Fungiform Density

Following euthanizing, mouse tongues were excised and the anterior two-thirds of the mouse tongue was fixed in 4% PFA for at least 24 hours before staining with 1% methylene blue for fungiform density analysis. The fungiform papillae appear as lighter blue dots on a background of blue stained tongue epithelia and were counted using ImageJ, in a region of interest representing a 1x1 mm square.

Taste Bud Isolation and RNA Extraction

Taste buds from the circumvallate papillae were isolated from mice at ~10 weeks old after Lickometer testing and one week wash out of NC and water *ad libitum*. Mouse tongues were freshly excised following euthanization with CO_2 and cervical dislocation. The isolated tongue was immediately immersed and rinsed in Normal Tyrode's solution (135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM MgCl, 5 mM $NaHCO_3$, 10 mM HEPES, 10 mM Glucose, 10 mM Sodium Pyruvate; pH 7.4). The sublingual epithelium surrounding the circumvallate papillae was injected with enzyme cocktail and then incubated in Ca^{2+} free Tyrode's solution (135 mM NaCl, 5 mM KCl, 20mM EGTA, 10 mM HEPES, 5 mM BAPTA (1,2-Bis (2-aminophenoxy) ethane-N, N, N, N-tetraacetic acid tetrapotassium salt), 10 mM Glucose, 10 mM Sodium Pyruvate; pH 7.4) for 15 minutes at room temperature (Dispase II 2.5 mg/ml, Collagenase A 1mg/ml, Elastase 0.25 mg/ml, and DNaseI 0.5mg/ml in Normal Tyrode's solution). The top epithelium was carefully peeled away from the tongue and individual taste buds were collected using a glass fired polished micro pipette with coated in 0.2% PVP to prevent cells from sticking

to the glass. Additionally, a piece of the epithelium posterior to the circumvallate papillae was cut out after taste bud collection, known as the “non-taste” area was collected as a non-chemosensory control epithelial tissue, and processed in parallel. All samples were immediately lysed and processed for RNA extraction.

RNA extraction, reverse transcription, and determination of gene expression

Total RNA was extracted using Absolutely RNA Nanoprep Kits for taste samples and RNA Microprep Kits for non-taste samples (Agilent) and used as template for cDNA synthesis with qScript cDNA SuperMix (Quanta Bio). Quantitative real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) was run on a QuantStudio 6 Flex Real-Time PCR System (Thermo). PLCβ2 enrichment over non-taste samples was used as a positive control for taste cells. Relative quantification was performed in triplicates using QuantStudio PCR Software, based on the $2^{-\Delta\Delta Ct}$ methods. Beta-Actin was used as the endogenous housekeeping gene for normalization of genes of interested (Table 1).

Protein	Gene	Forward 5'-3'	Reverse 5'-3'	Product size (bp)
β-actin	*Actb*	caccctgtgctgctcacc	gcacgatttccctctcag	328
PLCβ2	*Plcb2*	gagcaaatcgccaagatgat	ccttgtctgtggtgaccttg	163
T1R1	*Tas1r1*	ctggaatggacctgaatggac	agcagcagtggtgggaac	185
T1R2	*Tas1r2*	aagcatcgcctcctactcc	ggctggcaactcttagaacac	114
T1R3	*Tas1r3*	gaagcatccagatgacttca	gggaacagaaggacactgag	283
Gαgus	*Gnat3*	gcaaccacctccattgttct	agaagagcccacagtctttgag	286
Gα14	*Gna14*	attagctacttcccagagtacaca	gctcagatcaccctctgtct	256

Table 1. Primer sequences used for qPCR analysis.

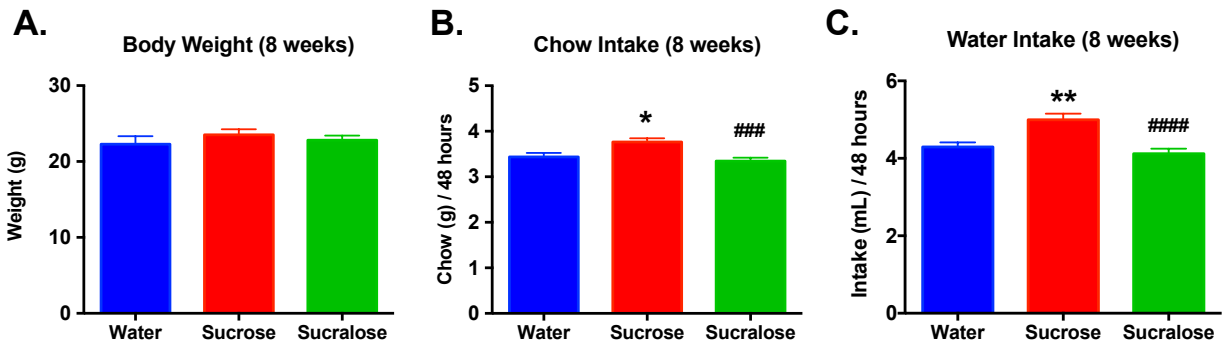
Results and Discussion

In a study of maternal sucrose consumption in rats, researchers found that a 10% sucrose solution provided ad libitum was sufficient to impair maternal metabolism, but the offspring showed no similar detrimental effects (Kendig et al 2015). Thus, we did not expect to find any adverse effects to metabolism in the offspring (i.e. glucose tolerance). Recent studies by Zhang et al (2011) showed that maternal ad libitum consumption of acesulfame-K led to sustained and detectable levels of acesulfame-K in the mouse amniotic fluid during pregnancy as well as the milk during lactation. This work suggests that maternal acesulfame-K exposure contributed to programming a preference for sweet taste in the offspring. In our studies we found no difference in sucrose and sucralose consumption due to maternal sucrose and sucralose exposures respectfully. A key difference between our studies and the work by Zhang et al (2011) is that we transformed human relevant daily intake values for sucrose and sucralose and determined daily treatment rations to the mothers. Zhang et al (2011) provided dams with ad libitum acesulfame-K resulting in approximate consumption of 400-600 mg/kg body weight each day. This dose comes out to be more than 20-fold higher than the maximum average daily intake (ADI) of sucralose, which is15 mg/kg body weight (Mattes and Popkin 2009).

Adult offspring outcomes at 8 weeks of age

Adult offspring show no significant difference in body weight between the two sweet maternal treatment groups compared to control (Figure 1A). One-way ANOVA reveals that baseline water consumption over 48 hours differed between treatment groups ($p<0.0001$). Specifically, the maternal sucrose offspring consumed more water

than the controls (p<0.01, Figure 1C). The maternal sucrose offspring consumed more normal chow diet on average compared to the water controls (Figure 1B).



	Maternal Treatment			
	Water	Sucrose	Sucralose	ANOVA p=
Body Weight (g)	22.3 ± 1.04	23.5 ± 0.74	22.8 ± 0.60	0.5511
Intake over 48 hours				
Chow (g)	3.44 ± 0.09	3.76 ± 0.08*	3.35 ± 0.07###	0.0007
Water (g)	4.30 ± 0.12	5.00 ± 0.16**	4.12 ± 0.13####	<0.0001

Figure 1. Baseline measurements in offspring of each treatment at 8 weeks of age (n=13-21). Values are expressed as mean ± SEM. Data were analyzed by one-way ANOVA and followed by post-hoc Tukey (when ANOVA p < 0.05). */**/***/**** when p<0.05/ 0.01/ 0.001/ 0.0001 compared to water group, ###/#### when p<0.001/0.0001 compared to sucrose group.

Brief-access sweet taste response

We aimed to test sweet taste responses in the offspring as adults (8 weeks) to identify if maternal sweet supplementation resulted in any long-term changes in sweet taste responses. We found the offspring of dams supplemented with sucrose and sucralose to show no difference in sucrose and sucralose taste behavior tests (Figure 2). Additionally, when treatment groups were analyzed based on sex, no differences were revealed (analysis not shown).

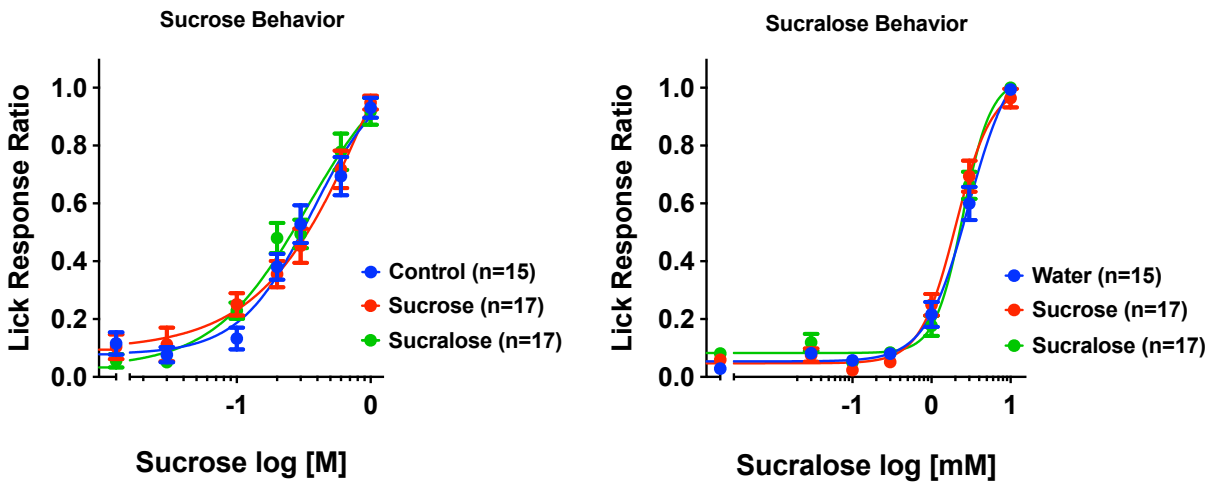


Figure 2. Adult offspring of maternal sucrose and sucralose treatments show no change in sweet taste response to sucrose ($F_{8,331}$=0.8821, p=0.5317) and sucralose ($F_{8,331}$=1.489, p=0.1598) when compared to controls.

Two-bottle testing with sucrose and sucralose

Although we did not see differences in sweet response at the taste bud level using Lickometer testing, we further characterized any changes in sweet intake behavior using two-bottle testing. This method allows us to determine if post-ingestive effects or other downstream processes are altered in the adult offspring. A change in sucrose or sucralose preference or intake could indicate that reward signaling or motivation may be altered in these mice. However, similar to Lickometer, we did not record any difference in sucrose or sucralose intake between the treatments and control (Figure 3 and 4). Previous studies have shown increased sucrose preferences in mice exposed to sucrose in early life after weaning (Sato et al 1991); however, we could not elicit these same responses in our exposure that occur even earlier during the perinatal period.

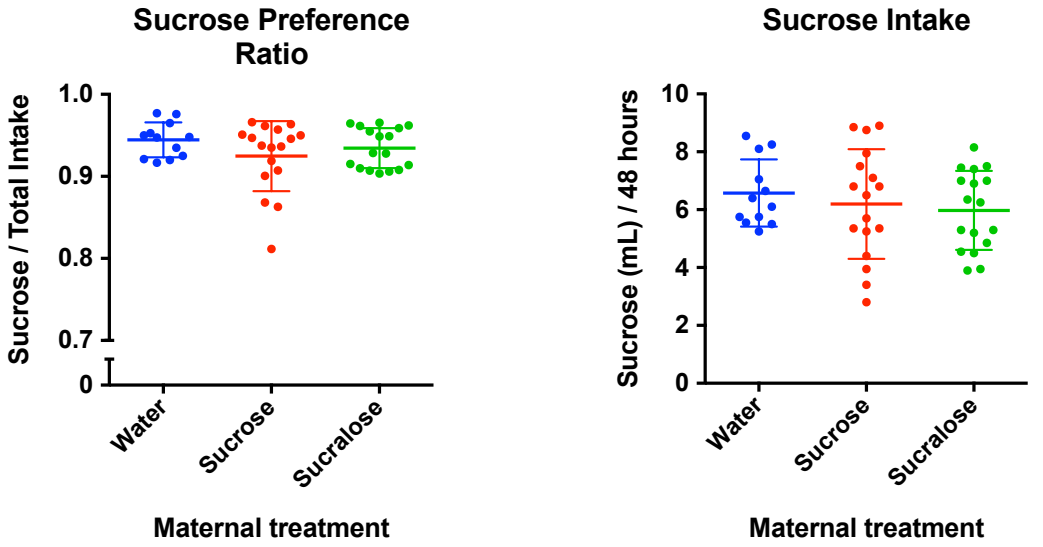


Figure 3. Adult offspring of maternal sucrose and sucralose treatments show no significant difference (one-way ANOVA, P=0.2657) in sucrose preference or total sucrose intake (one-way ANOVA, P=0.5883) when compared to the controls. Two-bottle sucrose choice was tested using a 0.03 M sucrose solution. Adult offspring of maternal treatment with water n=12, sucrose n=17, and sucralose n=17.

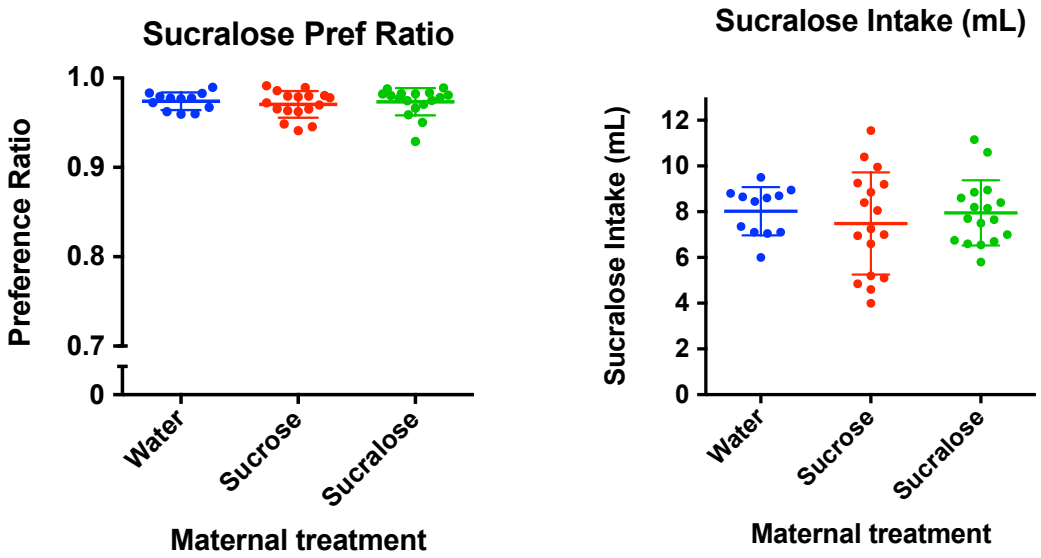


Figure 4. Adult offspring of maternal sucrose and sucralose treatments show no significant difference (one-way ANOVA, P=0.7404) in sucralose preference or total sucralose intake (one-way ANOVA, P=0.6324) when compared to the controls. Two-bottle sucralose choice was tested using a 1 mM sucralose solution. Adult offspring of maternal treatment with water n=12, sucrose n=17, and sucralose n=17.

Fungiform density

We counted fungiform density on the anterior tip of the tongues of mice from each treatment group as a measure of taste sensitivity (Figure 5). Increased fungiform density has been correlated with increased taste sensitivity at least in humans (Miller and Reedy 1990). Analysis of the fungiform density reveals no difference between the control and treatment groups.

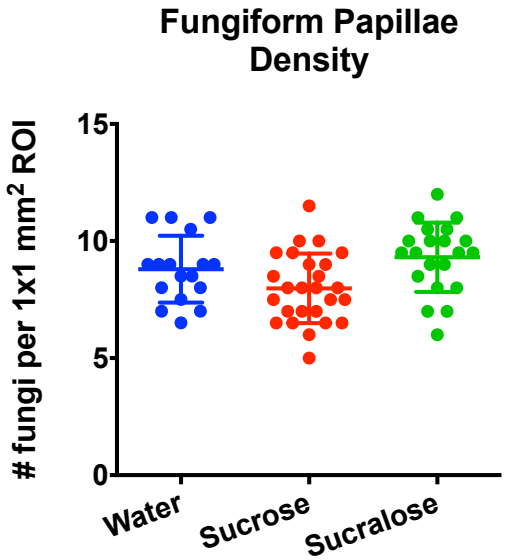


Figure 5. The adult offspring of maternal sucrose and sucralose treatments show no significant difference between the water and sucrose treatment as well as the water and sucralose treatment groups. A one-way ANOVA amongst the three groups shown is significant (P=0.0107); although, the significant difference lies between the sucrose and sucralose maternal treatment groups. Adult offspring of maternal treatments with water n=17, sucrose n=26, and sucralose n=21.

Taste gene expression

We used quantitative RT-PCR to examine gene expression in the taste buds of adult offspring of the three maternal treatment groups. Particularly we were interested in the T1R receptor family used for detection of sweet and umami tastes, and PLCβ2 and g-alpha subunits 14 and 3 utilized in taste receptor signaling. We found that maternal

treatment with sucrose and sucralose had no effect on the adult offspring taste bud gene expression (Figure 6).

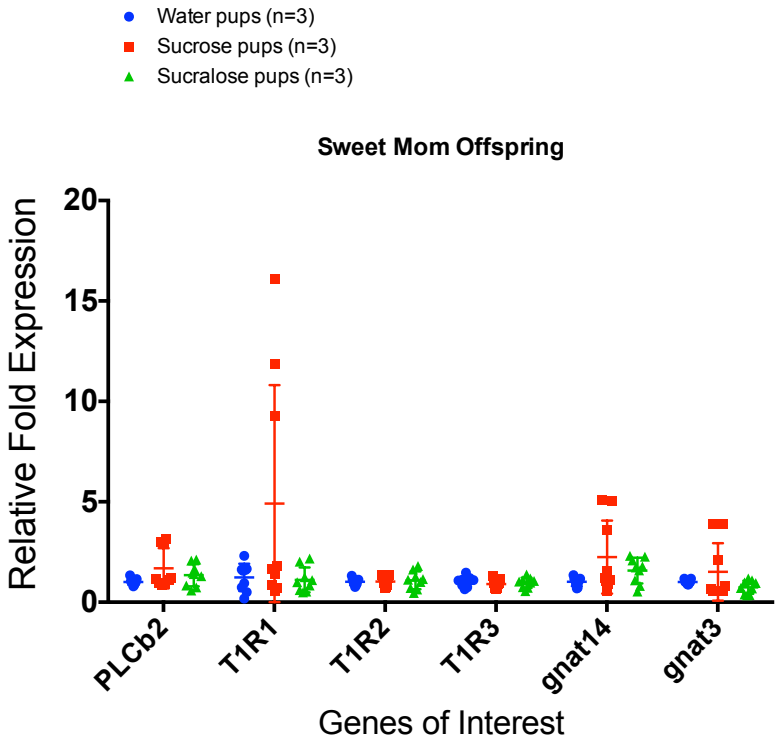


Figure 6. Gene expression analysis of taste bud samples collected from adult offspring of the different treatment groups. A multiple one-way ANOVAs reveal no significant differences amongst the treatment groups. Data are shown as means ± SEM and each biological sample was run in triplicate. The result of one mouse in the sucrose treatment group is likely an outlier and result of contamination during the RNA extraction steps.

Conclusions

Overall, results from this study suggest that sucrose and sucralose consumption at human-relevant levels during pregnancy and lactation do not result in significant long-term effects on the offspring's taste system, at least within the statistical power offered here. Adult offspring at 8 weeks of age for both the sucrose and sucralose maternal supplementation groups showed no change in sucrose or sucralose responses. No effect of maternal sweet supplementation was detected at the taste bud level (i.e.

fungiform density and taste bud gene expression remained unchanged). Our findings here suggest that the sweet taste system may not be as plastic and easily programming through the maternal diet as we may think. Our study suggests a sucrose or sucralose imbalance in the maternal diet does not have a lasting effect on the offspring taste system, although it is possible that short-term changes to the offspring taste system could occur in early life (post-weaning), which would not have been captured in this study.

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CHAPTER 6

Conclusions and Recommendations

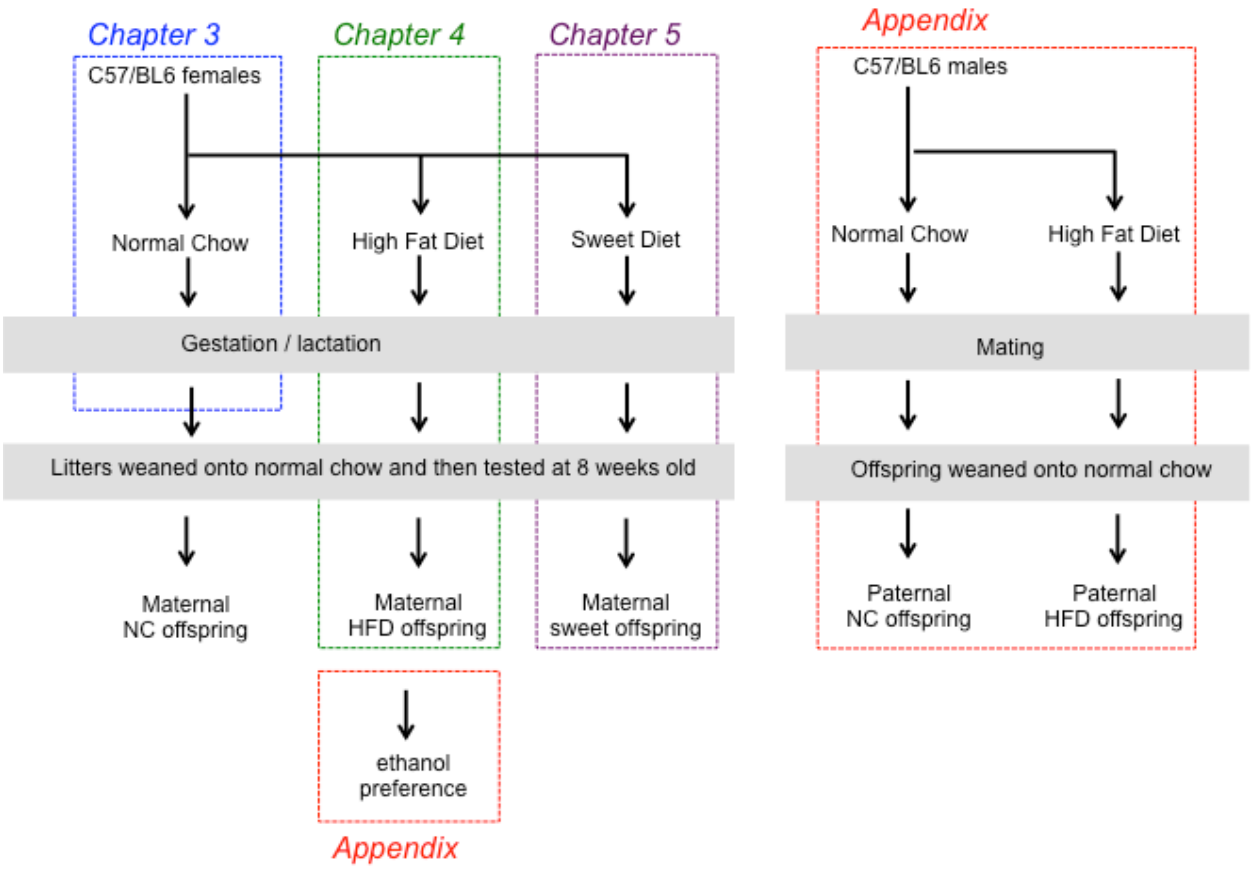


Figure 1. Overview of chapters

In Chapter 4 we studied the long-term impact of maternal HFD feeding on the taste system of the offspring in studies of taste and feeding behavior, gene expression, and taste bud morphology. The offspring of maternally obese dams showed an enhanced response to sucrose. These offspring had increased expression for subunits T1R2 and T1R3 that form the sweet receptor heterodimer and showed increased consumption of 0.1 M sucrose, 1.0 mM sucralose, and high-fat diet relative to their lean control counterparts. Behavioral changes in the adult offspring induced by maternal

obesity correlate with increased expression of sweet taste receptors in the taste buds, which may drive the increased preference for palatable foods. The results suggest taste bud function can be altered through maternal obesity, which adds to our understanding of how maternal obesity contributes to the offspring's risk of developing obesity. Future investigations should examine offspring taste behavior over the course of development or during advanced age (i.e. 6 months) as opposed to only 8 weeks as was done here. Further studies could investigate possible epigenetic regulation of the taste receptors in the adult offspring, by supplementing the maternal diet with methyl donors to reverse the hypothesized DNA hypomethylation of taste receptors in the adult offspring. Finally, the long-term effects of maternal HFD feeding on the adult offspring can be further investigated by studying the taste system in the F2 to determine if the phenotype has transgenerational persistence suggesting evidence of long-term maternal programming.

The findings here prompted the question - When does the maternal HFD treatment need to happen to cause this change in sweet taste? More specifically, is HFD feeding before or after mating more important for causing the shift in sweet sensitivity in the offspring? Studies of intermediate maternal treatments mCH and mHC revealed that the maternal HFD before *and* after conception was necessary to produce enhanced sweet sensitivity in the adult offspring. This suggests that the maternal HF treatment needs to be in place before conception to generate excess weight in the mothers and then be sustained through the gestation and lactation period.

A second question from these findings was - Why do we see a change in sweet sensitivity when the moms were fed HFD – could it be the sucrose in the HFD that’s actually producing this effect? In Chapter 5 we performed similar studies, but this time

using sucrose and sucralose at doses relevant to human consumption as well as the sucrose amounts consumed during maternal HFD treatments. The adult offspring at 8 weeks of age for both the sucrose and sucralose maternal supplementation groups showed no change in sucrose and sucralose responses. Additionally, there was no effect detected at the taste bud level (i.e. fungiform density and taste bud gene expression remained unchanged). Overall, the findings suggest that sucrose and sucralose consumption during pregnancy and lactation do not produce long-term effects on the adult offspring taste system. The sweet taste system may not be as plastic and easily programmable through the maternal diet as we may think. Future work could focus on the taste system of weanlings instead of at adulthood as in this study. Sucrose and sucralose supplementation may affect early taste functions prior to physiological maturation that would not have been captured in this study. To my knowledge, few studies have considered what happens to the regulation of sweet taste receptors after considerable time of exposure (i.e. sucrose or sucralose feeding in adult mice for 5 weeks and then studying the taste buds a week later). Because a viable taste cell culture system has yet to be formulated, an *in vivo* study such as this is necessary.

In Chapters 2 and 3 we focused on the topic of the maternal diet and pregnancy. We know from rodent studies that, if given the choice between chow and a junk food diet, pregnant mice overconsume the junk food diet. Taste changes during pregnancy have been reported for decades; however, a longitudinal analysis of the exact changes in taste before, during, and after pregnancy has yet to be carried out with humans (as reviewed in Chapter 2). Integrated results from animal studies of pregnancy demonstrate the utility of animal models for studying taste changes during pregnancy.

Results from existing human studies suggest a change in taste during the 1st trimester, usually a small decrease in taste function. This would lead one to assume that this is due to the sudden increase in hormones at the beginning of pregnancy. We propose that researchers consider other paradigms to explain modulation of the taste system in pregnancy. Integrated animal studies to study the mechanisms underlying taste changes during pregnancy may help in advancing our understanding of feeding behavior during this important period. To this end, in Chapter 3, we investigated taste behavior, taste gene expression, and gustatory morphology in female mice before, during, after pregnancy.

In a mouse model using brief-access Lickometer testing, we show that sweet responses are diminished during pregnancy. The underlying mechanisms resulting in altered sweet taste responses during pregnancy remain to be clarified, but we speculate that the decrease in taste bud number in the circumvallate papillae may precipitate changes in taste response. In this study we focused on sweet taste. Future studies could investigate other tastes such as bitter, umami, and fat. The act of gustation requires a taste stimulus to activate receptors found at the taste cell membrane, which then activates cellular signaling pathways to eventually transduce the signal to the brain to be perceived as gustation. Based on our current findings, future investigation could focus on other transcripts involved in sweet signal transduction; for example, by using microarray or RNA-sequencing.

The appendix contains a pilot study that extends from the findings in Chapter 4 showing that mHH adult offspring have increased ethanol preference and intake. Additionally, paternal HFD treatment does not elicit a change in HFD preference or

intake in the adult offspring (Appendix).

Animal studies provide the potential to evaluate intervention strategies. Studies designed to increase our understanding of what factors alter the taste system and thus in turn influence feeding behavior may help us to understand the link between overnutrition in early life and later disease risk in adulthood. This research is one of just a few studies to characterize taste surrounding the critical physiological state of pregnancy and the first and only study to do so in a way that informs the long-term effects of maternal HFD on the offspring taste system.

Appendix

Does paternal HFD lead to increased HFD preference or intake in the adult offspring?

Background

Evidence for paternal taste transmission through epigenetic means

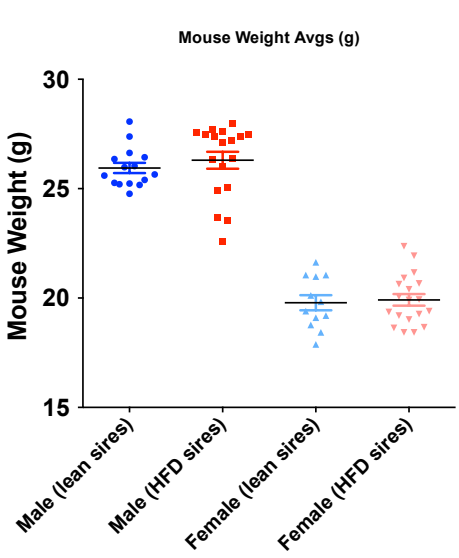
Paternal chronic HFD has been associated with alterations in offspring glucose metabolism (Ng et al 2010) and paternal low protein diet with DNA methylation at several metabolically relevant loci in the offspring's liver (Carone et al 2010). In conjunction with olfactory stimuli, parental traumatic exposure was inherited by the offspring in association with hypomethylation on the related olfactory receptor gene when examined in paternal sperm (Dias and Ressler 2013). Studies on fetal ethanol exposure in a rodent model found increased taste-mediated acceptability of ethanol and quinine but not sucrose. This suggests an epigenetic mechanism by which maternal chemosensory patterns can be transferred to the offspring (Youngentob and Glendinning 2009). Interestingly, male offspring of ethanol-exposed sires show reduced ethanol preference and consumption (Finegersh and Homanics 2014).

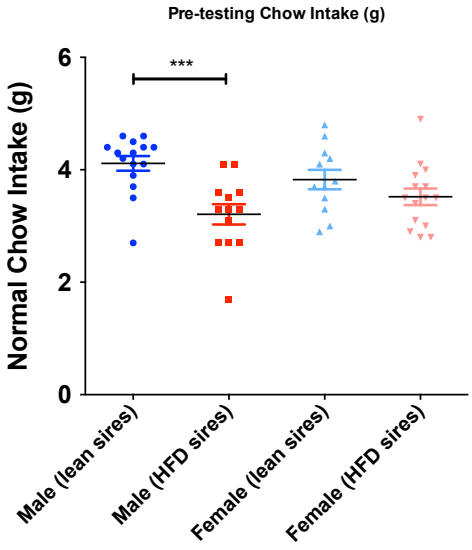
Study goals

This study focused on whether HFD treatment in sires could produce an increased preference for HFD in the adult offspring, as similarly seen in experiments of maternal HFD treatment. If this had been a significant result, we would have followed up with brief-access Lickometer testing to better characterize the taste change to linoleic acid (tastant for fat) and sucrose (tastant for sweet) and immunohistochemistry to investigate taste morphology.

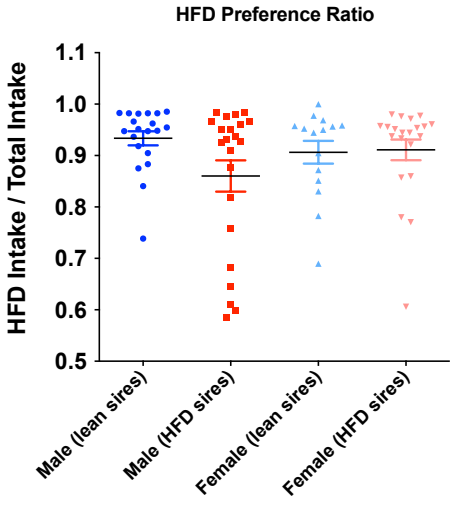
Results

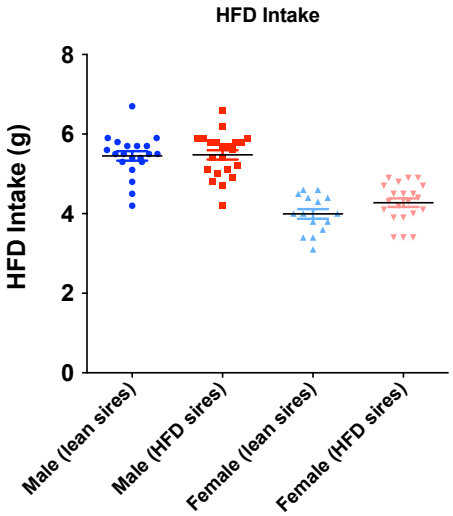
We found no difference in body weight between the adult offspring (8 weeks old) of lean and obese sires. Prior to any testing, the adult male offspring of HFD sires consumed significantly less chow than their lean counterparts. The adult female offspring of HFD sires also consumed less chow, although not statistically significant.



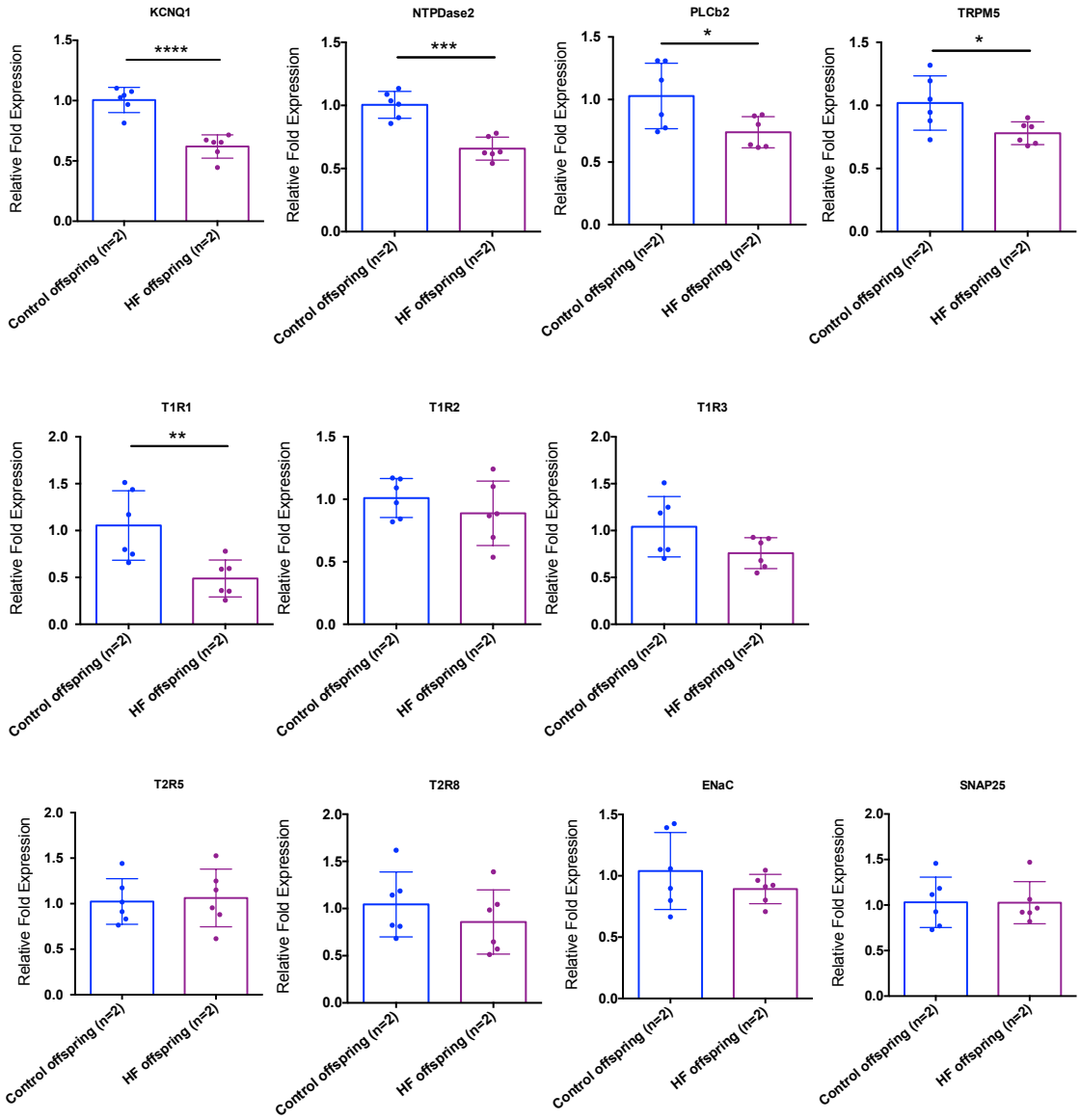


No difference in HFD preference or intake between the two paternal treatment groups.





Taste buds were also isolated and real time RT-PCR was used to compare transcript activity at the taste bud level in the two groups. Although a number of taste genes were down regulated, these results are preliminary and only consist of 2 biological replicates. Since no differences were observed in HFD intake of preference between the two groups, it is difficult to speculate how these changes in gene expression could alter taste perception and ultimately feeding behavior. Data is shown as mean ± SEM and t-tests were performed for statistical analysis.



In human studies of parental macronutrient and energy intakes during pregnancy and the effects on the offspring at 10 years of age, researchers found that the maternal diet (i.e. protein, fat, carbohydrates) in pregnancy were positively associated with child dietary intakes for the same nutrients, while paternal dietary intake was not (Brion et al 2010). Thus, it is unsurprising that we did not see any long-term effects on the adult offspring taste system due to paternal HFD feeding prior to mating.

Conclusions

The goal of this study was to determine if paternal HFD treatment prior to mating resulted in adult offspring with increased preference for HFD. In this pilot study, we found no significant different in HFD preference or intake between the two groups. Surprisingly, the data shows that adult male offspring of paternal HFD exposure consume less chow than their lean counterparts although their body weight is comparable. This may be a result of an altered metabolism (Ng et al 2010). From the mRNA expression data we see a significant decrease in the T1R1 receptor subunit important for umami responses; however, based on our limited pilot data, it is difficult to speculate how a decrease in the T1R1 subunit could affect taste responses and ultimately dietary behavior.

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Appendix

Adult offspring of maternal HFD and their ethanol preference and intake

Background

Gestational ethanol exposure has long-term effects on the offspring taste system, by making ethanol taste and smell better than how it is typically perceived by controls with no ethanol exposure (Youngentob and Glendinning 2009). Follow-up studies revealed that the maternal ethanol exposure also attenuated capsaicin-like burning oral sensations, contributing to increased ethanol avidity (Glendinning et al 2012). These findings are consistent in humans where fetal ethanol exposure is highly predictive of adolescent ethanol use and abuse. Sweet sensitivity has been correlated with less obvious phenomena such as drug seeking behavior, impulsivity, and risk taking behavior (Dess et al 1998, Perry et al 2007, Anker et al 2008, Carroll et al 2008). Interestingly, the T1R3 sweet receptor subunit is necessary for gustatory neural responses to ethanol and for the oral ethanol preference found in rodents (Brasser et al 2010). Given the link between sweet taste and ethanol consumption we hypothesized that the mHH offspring that show increased sucrose and sucralose consumption would also consume more ethanol compared to controls.

Methods

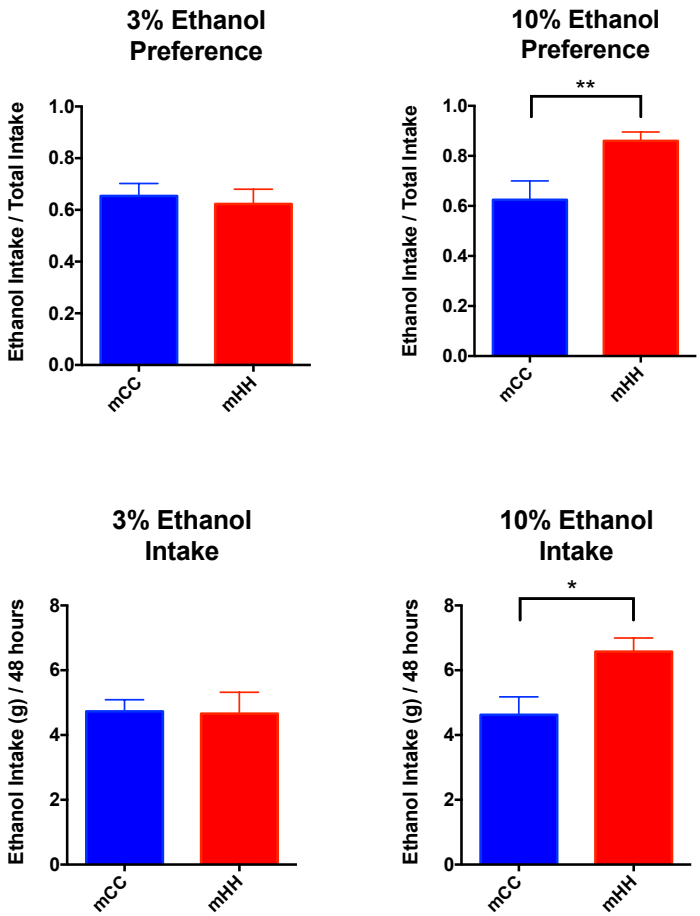
Two Bottle Testing – 3% and 10% Ethanol

Following two diet testing with chow and HFD, mice were trained to consume water from two bottles for 48 hours. First mice were tested with one bottle of water and

one bottle of 3% ethanol and for the second test 10% ethanol. Mice were given simultaneous access to the two bottles and consumption was measured over 48 hours. Bottle order was random and side swapped after 24 hours. Ethanol preferences were calculated as a percentage of ethanol intake divided by total fluid intake. Methods were adapted from Bachmanov et al 2001.

Results

In tests with 3% ethanol, there was no significant difference between ethanol consumption between the two groups. Even when the data was stratified by sex there



was no difference in 3% ethanol preference or intake (data not shown). Interestingly, the mHH adult offspring consume more 10% ethanol than the controls. This effect was not sex specific based on two-way ANOVA analysis in which there was not significant maternal treatment x sex effect (data not shown).

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Appendix

C-Kit receptor is expressed in sweet and umami responding T1R3 adult taste cells

Abstract

Previous studies have described a number of protein tyrosine kinases (i.e. epidermal growth factor receptor, ErbB2, ErbB3, and c-kit) to be expressed in taste bud cells, innervating nerves, and developing papillae. Here we show that c-kit positive cells are exclusively co-localized with T1R3 adult taste cells using immunofluorescence tissue staining. This suggests that c-kit+ cells are mostly made up of T1R3+ taste cells, which respond to sweet and umami, but not bitter. Using calcium imaging, we show that the majority of c-kit-eGFP+ taste cells respond to sweet and umami tastants. The data indicate that the control of c-kit receptor activation may be important for proper mature taste cell formation and may aid in the maintenance of this specific mature taste cell subpopulation. Further characterization of the c-kit receptor and its functional role in taste may lead to better ways to culture taste cells and organoids and help elucidate mechanisms of taste cell regulation.

Kit (NM_001122733) receptor is also known as CD117, c-Kit

Kitl (NM_013598.2) ligand is also known as Stem Cell Factor (SCF), Kit ligand

Introduction

Taste bud cells, unlike other sensory cells like that of the retina and inner ear, are in a constant state of renewal and turnover every ten to fourteen days (Beidler and Smallman 1965, Farbman 1980). C-Kit is a receptor tyrosine kinase that binds the kit ligand. This receptor-ligand pair interaction is known to be critical for survival and development of stem cells involved in hematopoiesis, pigmentation, and reproduction (Linnekin 1999).

KIT is expressed in taste buds of both developing and adult taste buds in the rat (McLaughlin 2000). Interestingly, KIT is known to control stem cell survival and development in bone marrow, skin, and the gut (Lennartsson et al 2005); however, in adult taste cells KIT in exclusively expressed in the adult taste cells (McLaughlin 2000). Genome-wide analysis of gene expression in primate (*Macaca fascicularis*) taste buds using laser capture microdissection found the receptor-ligand pair KIT and KIT ligand (Hevezi et al 2009). In macaque taste tissue sections, KIT was expressed exclusively in TAS1R1 (sweet and umami receptor) circumvallate taste cells using *in situ* hybridization, suggesting that KIT may modulate sweet and umami cell differentiation and development (Hevezi et al 2009).

The W/W^v mutant mouse has point mutations at the white spotted locus that results in an abnormal kit gene and decreased kinase activity (Galli et al 1993). These mice show extensive loss of gastrointestinal interstitial cells of Cajal, have altered meal patterns, and altered CCK sensitivity despite no apparent different in daily food intake and body weight (Chi and Powley 2003). Patients treated with sunitinib malate (trade name Gleevec), a potent inhibitor of multiple tyrosine kinase receptors report taste

alterations (Adams and Leggas 2007, Goodman et al 2007). Here we aimed to localize KIT and KIT ligand in mouse taste buds. The functional role of KIT in taste cells remains to be determined. Thus, the aim of this study was to determine which taste cell type c-kit is co-localized.

Methods

Animals

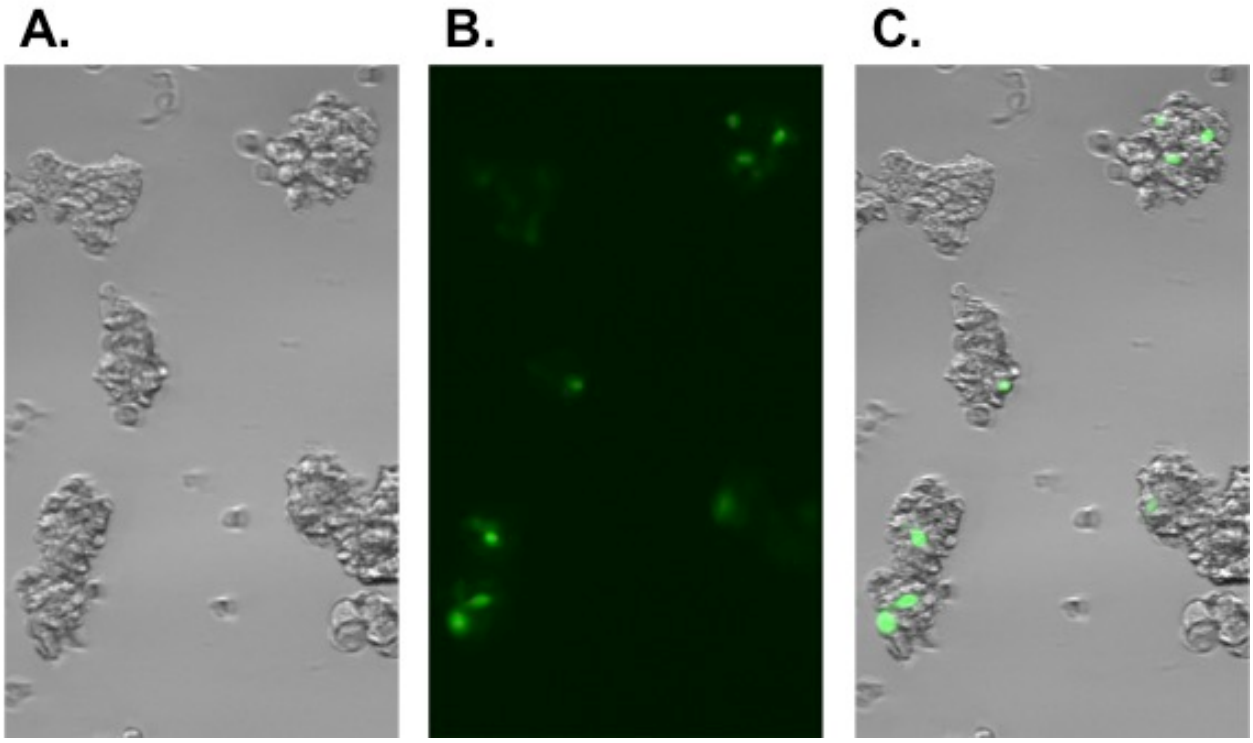
We used in-house bred C57BL/6 mice (originally purchased from Jackson Laboratory) for immunohistochemistry. To carry out calcium recordings of taste cells, we used ckit-eGFP mice. These mice were a gift from the lab of Michael I Kotlikoff, but are commercially available from Jackson Laboratory as B6;D2-Tg(RP24-330G11-EGFP)1Mik/J, strain code: 025122. These mice were bred and genotyped as directed by Jackson Laboratory instructions. Within each strain, animals were randomly selected for experiments. Experiments were conducted with approval by the Institutional Animal Care and Use Committee at Cornell University.

Taste bud isolation

Mouse tongues of ckit-eGFP mice were freshly excised following euthanization with CO_2 and cervical dislocation. The isolated tongue was immediately immersed and rinsed in Normal Tyrode's solution (135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM MgCl, 5 mM $NaHCO_3$, 10 mM HEPES, 10 mM Glucose, 10 mM Sodium Pyruvate; pH 7.4). The sublingual epithelium surrounding the circumvallate papillae was injected with enzyme cocktail (Dispase II 2.5 mg/ml, Collagenase A 1mg/ml, Elastase 0.25 mg/ml, and DNaseI 0.5mg/ml in Normal Tyrode's solution) and then incubated in Ca^{2+} free

Tyrode's solution (135 mM NaCl, 5 mM KCl, 20mM EGTA, 10 mM HEPES, 5 mM BAPTA (1,2-Bis (2-aminophenoxy) ethane-N, N, N, N-tetraacetic acid tetrapotassium salt), 10 mM Glucose, 10 mM Sodium Pyruvate; pH 7.4) for 15 minutes at room temperature.

The lingual epithelium was carefully peeled away from the circumvallate papillae, incubated a second time with enzyme cocktail as above, then washed in Tyrode's and individual taste buds collected using a glass fired polished micro pipette coated in 0.2% PVP. Taste buds were transferred to a shallow recording chamber, secured with Cell-Tak (BD Biosciences, San Jose, CA) and superfused with Normal Tyrode's following incubation with 5 uM Fura-2-AM for 60 minutes.



Isolated taste buds showing ckit-GFP+ cells within cell clumps.

Ca2+ imaging of taste cells

Tastant solutions were prepared using Normal Tyrode's: bitter (30 uM cycloheximide, 200 uM denatonium), umami (200 mM monosodium-glutamate), and sweet (5 mM sucralose). Images were taken every 2 seconds, and results presented as relative fluorescence: ΔF/F = ([F-F0]/F0). Photobleaching was corrected by plotting gradual decline of signal over time and stimuli applied more than 2 minutes apart to prevent desensitization (Caicedo et al 2000). Images were taken using an Olympus IX-71 microscope at 340 and 380nm, with a Hamamatsu Orca Flash 4.0 camera and Molecular Devices Metamorph imaging suite (Sunnyvale, CA).

Tissue preparation

One hour prior to euthanization, mice were injected with serotonin 5-HTP (2 mg / 25 g) to enhance immunofluorescence stain for 5HT+ taste cells. Mice were then humanely euthanization with CO_2 and cervical dislocation. Tongues were excised and then rinsed in PBS. The circumvallate papillae was carefully isolated with a sterile razor and fixed in 4% PFA at 4°C for one hour, cryoprotected in 30% sucrose overnight, and then embedded in OCT.

Immunofluorescence

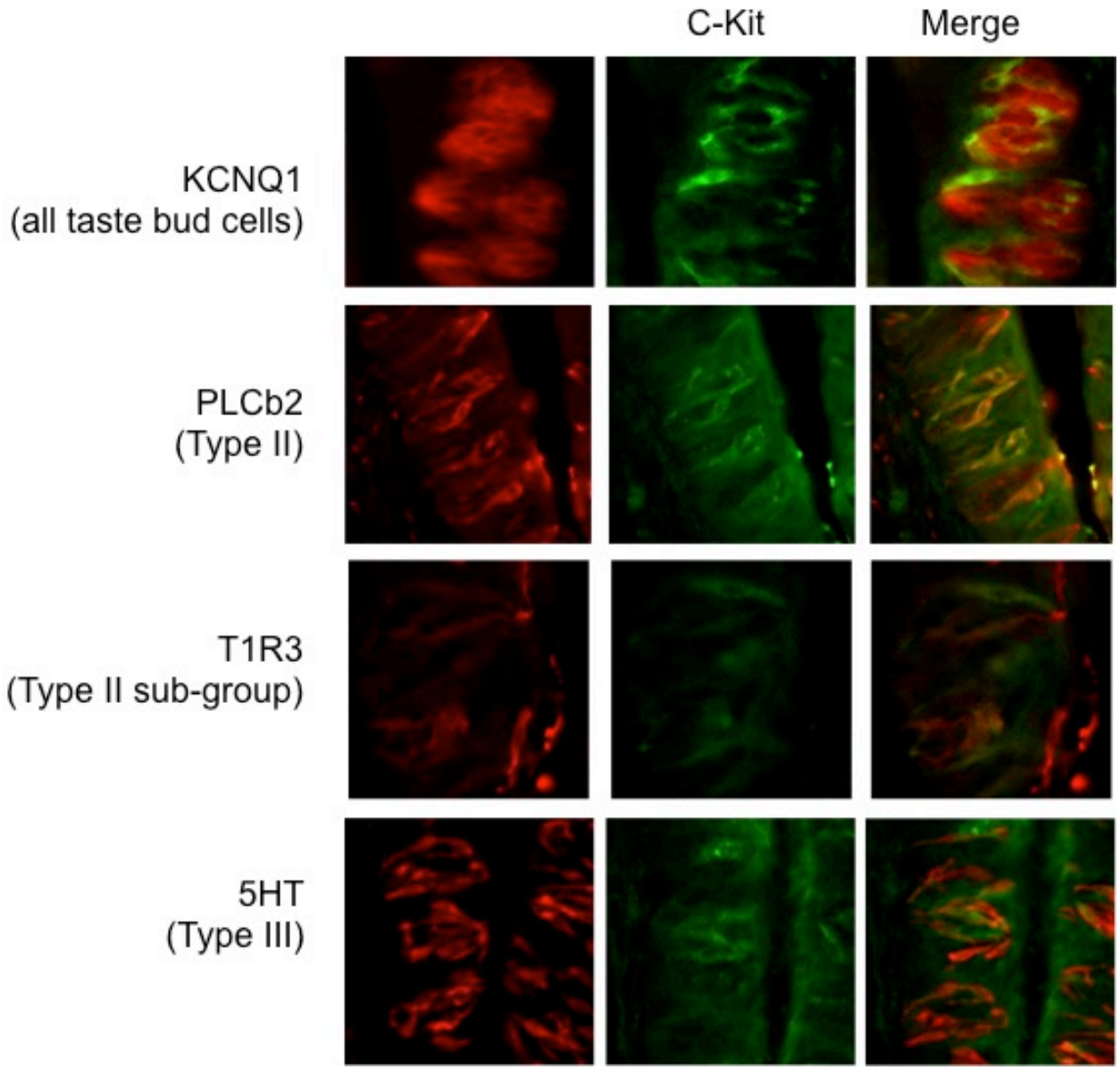
The 4% PFA fixed tissue was sectioned at 10 microns and stained with primary and secondary antibodies. Images were taken using an Olympus IX-71 microscope with a Hamamatsu Orca Flash 4.0 camera.

Antigen	Host	Vender	Dilution
NTPDase2	rabbit	J. Sévigny at Université Laval, Quebec	1:1000
PLCb2	rabbit	Santa Cruz Biotechnology	1:1000
PLCb2	goat	Santa Cruz Biotechnology	1:500
T1R3	goat	Santa Cruz Biotechnology	1:1000
KCNQ1	goat	Santa Cruz Biotechnology	1:1000
5HT	rat	Millipore	1:1000
Gustducin	rabbit	Santa Cruz Biotechnology	1:1000
c-kit	rabbit	Genemed Biotechnologies	1:1000
c-kit	goat	R&D Systems	1:1000
Ki67	rabbit	Thermo	1:1000
Sox2	goat	Santa Cruz Biotechnology	1:1000
SCF	rabbit	Santa Cruz Biotechnology	1:50
TRPM5	guinea pig	ER Liman at University of Southern California	1:5000

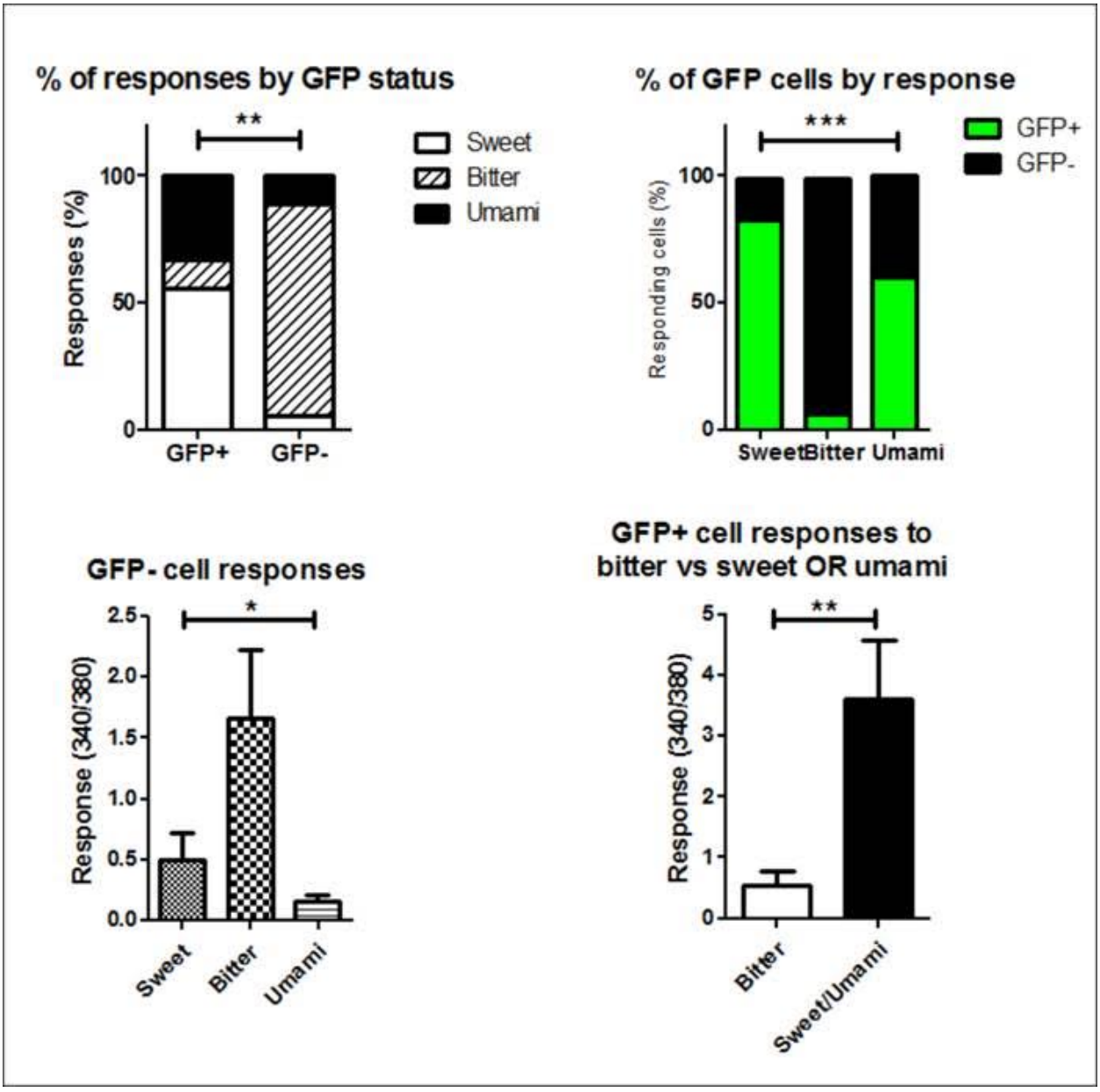
Results

We found c-Kit to be expressed in T1R3 sweet and umami taste cells in mouse tissue of the circumvallate papillae using immunofluorescence tissue staining. With calcium imaging of individual taste cells, we show that the majority of c-kit-GFP+ taste cells respond to sweet and umami, but not bitter.

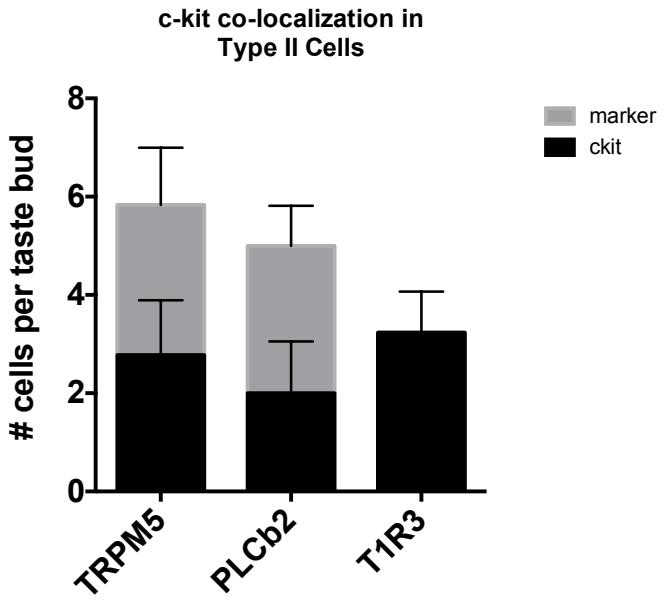
Additionally, SCF was localized in the nuclei in all Type I, II, and III taste cells that make up the taste bud as well most of the non-taste cells in the top epithelium. Co-expression of c-kit and SCF in the same cells as the c-kit receptor, suggests a self-regulation (Reber et al 2006). In agreement with existing literature (Biggs et al 2016), we found Kit and Kitl mRNA expression in mouse taste samples.



C-kit stain is co-localized with T1R3.



The majority of c-Kit-GFP+ cells respond to sweet and umami, but not bitter. This suggests that c-kit+ cells are mostly made up of T1R3+ taste cells, which respond to sweet and umami, but not bitter.

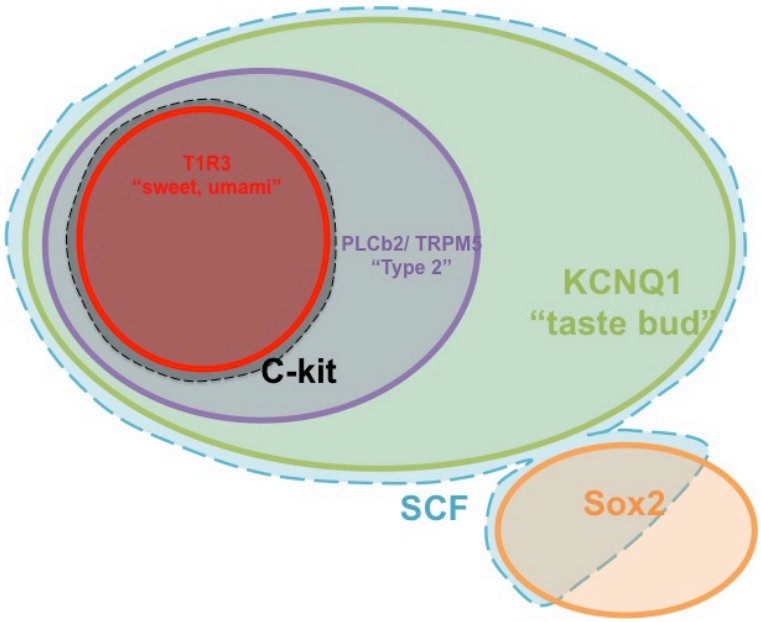


C-kit stain is exclusively co-localized with T1R3, but not Gust cells that mark bitter responding cells.

Discussion

Previous work by McLaughlin (2000) has shown neuronal staining by cKIT in taste structures during late embryonic and early postnatal development in mice. By P10, the neuronal staining is almost disappeared, yet cKIT staining remains in individual taste cells. Here we show that this taste specific c-Kit stain remains past P10 in adult mice, and that c-Kit expression is exclusively co-localized in T1R3 taste cells. Patients taking imatinib mesylate, a selective KIT receptor inhibitor, as a disease treatments have reported taste disturbances (Liu et al 2013). This suggests that the control of cKIT receptor activation may be important for proper mature taste cell formation and may aid in the maintenance of a specific mature taste cell subpopulation, similar to the role of BNDF in the paper by Huang et al (2015). Pancreatic cells express T1R3 receptors (for nutrient sensing) and share common signaling proteins with the taste system. Studies of pancreatic β-cells suggest c-kit and its ligand serve important roles in pancreatic islet

development by promoting islet cell differentiation and proliferation (Krishnamurthy et al 2007). Activation of cKIT has been found to stimulate a wide array of signaling pathways including MAPK, PI3K, and JAK/STAT. Given the complexity of signaling pathways and tendency to work as a network, we also set out to determine which if any taste cells express the c-Kit receptor ligand stem cell factor (SCF). We found SCF to at least be localized in the nuclei of all adult taste cells within the taste bud. This may suggest an autocrine signaling by the T1R3 cells and paracrine signaling by the surrounding taste cells. Because taste cells are highly specialized, and an adequate taste cell culture system has yet to be established, a further investigate of the role of c-Kit in taste buds are warranted.



Venn diagram showing co-localization of c-kit within the T1R3 subgroup of Type II cells and SCF in all taste cell nuclei.

Conclusions

Further characterization of KIT and KIT ligand and their functional role in taste may lead to better ways to culture taste cells and organoids and help elucidate mechanisms of taste cell turnover. Studies of olfactory tissue reveal that adult c-kit+ progenitor cells are important for olfactory epithelial renewal and tissue maintenance of the adult neuroepithelium (Goldstein et al 2015, Goss et al 2016). Since KIT KO mice are embryonically lethal, future studies could focus on the short and long-term effects of c-Kit inhibitors on the taste cells, particularly the T1R3 cells, and turnover rates to determine whether c-Kit is critical for adult taste cell maintenance. For example, a c-Kit antagonist such as imatinib mesylate (Gleevec), could be administered directly to the tongue and/or via oral gavage to bypass oral exposure, then IHC could be used to investigate changes in T1R3 taste cell numbers in the taste buds. Since c-Kit is localized in adult umami taste cells, I hypothesize that their role may be for maintenance and that an inhibitor would decrease T1R3 cell numbers. If this is the case, behavioral studies could then be carried out to determine if umami is the only basic taste affected. If other tastes are diminished, this may suggest cross talk between the taste cells and that c-Kit activate regulations adjacent cells in addition to T1R3 cells.

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Abstract for the New York Pharmacology Society Meeting 2016
Presidential Symposium (oral presentation)

Murine taste bud molecular modulation suggests modification of sweet sensitivity during pregnancy

Ezen Choo[1], Robin Dando[2]: [1]Pharmacology, College of Veterinary Medicine, Cornell University, Ithaca, NY; [2]Food Science, College of Agriculture and Life Sciences, Cornell University, Ithaca, NY

An alteration in maternal intake during pregnancy permanently affects the metabolism, growth, and feeding behavior of the progeny, in both mice and humans. While much is known about how maternal diet affects offspring fitness, less is known about how gustation is involved in guiding and promoting food intake during this crucial period. Women have intense food cravings and exhibit altered taste preferences during pregnancy. However, the mechanistic details underlying these changes during pregnancy are presently unclear. We investigated taste changes in pregnant mice using brief-access taste testing and found decreased sensitivity to sucrose during the mid-late stages of pregnancy (comparison of logEC_{50} values, $F_{1,101}$=6.455, p=0.013). We hypothesize that altered taste preferences in parturition results from changes in the expression profile of the taste buds of the mother, and from circulating hormones acting on cognate receptors in taste. We performed qPCR to study taste receptor expression, a potential pathway for the modulation of taste signaling. The results indicate that the physiological changes induced by pregnancy influence the taste transcriptome, suggesting that taste modulation may be a useful strategy to enhance offspring fitness through maternal intake.

Research support from the Center for Vertebrate Genomics and Cornell University College of Agriculture and Life Sciences Startup Funds.

Abstract for the Association of Chemoreception Sciences 2016
(poster presentation)

A Reduction in Sweet Taste Sensitivity in Pregnant Mice Correlates with Decreased Sweet Receptor Expression

Ezen Choo[1], Robin Dando[2]: [1]Pharmacology, College of Veterinary Medicine, Cornell University, Ithaca, NY; [2]Food Science, College of Agriculture and Life Sciences, Cornell University, Ithaca, NY

An alteration in maternal intake during pregnancy can permanently affect the metabolism, growth, and feeding behavior of the progeny, in both mice and humans. While much is known about how maternal diet affects offspring fitness, less is known about how gustation is involved in guiding and promoting food intake during this crucial period. Humans display intense food cravings and exhibit altered taste preferences during pregnancy; however, the mechanistic details underlying these changes during pregnancy are presently unclear. We investigated taste changes in pregnant mice using brief-access taste testing and found decreased sensitivity to sucrose during the mid/late stages of pregnancy. We hypothesize that altered taste preferences in parturition result from changes in the expression profile of taste buds of pregnant mice. Following this, we examined taste receptor mRNA expression as a potential pathway for the modulation of taste preference. Of the sweet receptor subunits, T1R2 expression was decreased during late pregnancy, while sweet and umami receptor subunits T1R1 and T1R3 were unchanged. Interestingly, the bitter receptors T2R5 and T2R8 appeared unchanged during mid/late pregnancy despite previous reports of increased bitter sensitivity during pregnancy. Our results imply that the various physiological changes induced by pregnancy may influence the taste transcriptome, and resulting feeding behavior during parturition.

FUNDING ACKNOWLEDGMENTS
Cornell University College of Agriculture and Life Sciences Startup Funds
Center for Vertebrate Genomics

Presentation Preference: General Submission
System: Taste: periphery
Experimental Approach: Molecular Biology/Genetics; Animal Behavior

Keywords – sweet, taste, behavior

Abstract for the Experimental Biology Conference 2017
(poster presentation)

Maternal Obesity Increases Sweet Taste Response and Sweet Taste Receptor mRNA Expression in Adult Offspring

Ezen Choo[1], Robin Dando[2]: [1]Pharmacology, College of Veterinary Medicine; [2]Food Science, College of Agriculture and Life Sciences

Maternal body mass index and gestational weight gain predict future over-weight/ obese status in children and adolescents. Based on animal studies, in both rodents and non-human primates, maternal obesity predicts a preference for palatable foods in the offspring, suggesting an increased preference for foods rich in fat, sugar, and/or salt. In this study, we investigate whether the underlying basis for an increase in palatable food consumption in the offspring of maternally obese mice is due to a change in taste. Mice were fed a control or high fat diet before and during gestation/ lactation, with all offspring subsequently maintained on control diet after weaning; thus, the only experience with high fat diet for the offspring was through maternal exposure during early development. Taste response was assessed in offspring after reaching maturity, using brief-access taste testing. The female offspring of maternal obesity showed an enhanced response to sucrose ($F_{4,146}$=3.986, p=0.004), whereas the males did not ($F_{4,118}$=0.512, p=0.7271). We hypothesize that this enhanced response results from changes in the expression profile of taste buds for sweet taste receptors. We performed qPCR to assay taste receptor expression, and found that both subunits composing the sweet receptor heterodimer, T1R2 and T1R3, to be increased in the female offspring of obese dams compared to lean (T1R2 p=0.035, T1R3 p=0.00053). In male offspring of obese dams, T1R2 (p=0.000091) expression was also increased compared to lean, but not T1R3 (p=0.21). The results indicate that behavioral changes in the adult offspring induced by maternal obesity correlate with increased expression of sweet taste receptors in the taste buds, which may drive the increased preference for palatable foods reported in offspring of maternally obese mice. These findings highlight the importance of maternal health and the long-term impacts of maternal obesity on offspring health.

Research support - CVG

Timed Mating Protocol

This mating protocol, for C57BL/6 mice, was designed to ensure the researcher knew what day the female was fertilized. Thus, male and female mice are placed together for one night, checked for copulatory plugs, and the female is reweighed ~12 days later to monitor pregnancy.

1. Two days prior to mating, put soiled bedding from the male cage into the female cage. The male-soiled bedding will restart her menstrual cycle and prepare her for mating. In the wild, it is the female mouse that chooses the male.

2. On the day of mating, it is recommended that male and female mice be placed together in the afternoon (3-6PM) before the lights go out. Place the female(s) into the male cage. One male can be kept with multiple females overnight. Males that mate at least once before reaching 4 months of age have been shown to be more successful. A male that has never mated before 4 months may never produce a successful plug.

3. Leave a “sleepover” label on the cage of the female(s), so that the facilities staff does not think the mouse went missing.

4. The following morning, check for plugs before noon. It is recommend that you check for plugs early in the morning because they will dissolve or fall out. Lift the female tail and check for a plug. If no plug is present then probe the mouse for resistance as the plug may be deeper inside. If the probe goes in easily, then she is most likely was not fertilized. Weight the mouse for your records.

Notes:

-If you separate a male from his original litter, then he cannot be returned to be with other males because they will fight. That mated male will need to be housed singularly from now on.

-Typically, a pregnant female will gain 3-5 grams after 12 days. No significant weight gain usually means she is not pregnant.

-It's good to keep track of the male's plug rate & pregnancy success, as there is always the chance he may be infertile.

2 Bottle Testing

Run 2 tests, one side with the blank and the other with the tastant, and then average

1. Train mice to consume from two bottles by providing 2 licko bottles of regular/ ECRF water for at least 48 hours (i.e. 2 days of training).

2. Begin testing with vehicle on one side and tastant to test on the other side. Alternate starting positions for every other mouse.

3. After 24 hours, swap side positions. Bottles may need to be measured and refilled if levels are low. Some mouse strains have been shown to have side preferences.

4. After 48 hours, measure remaining liquid to calculate how much was consumed.

Notes

- Have a control cage with 2 tubes of water set up, to gauge how much is lost to handling and evaporation, and subtract this from the totals.
- Keep the same bottles and spouts for each mouse, or use new clean ones, as there may be pheromone issues etc.
- Each licko bottle can hold about 15 mL

Diet Preference Protocol

1. To acclimate, separate mice into individual cages 1 week prior to training. Provide water and chow ad lib.

2. Use the duel hopper cage topper and begin training mice to consume chow from both hopper sides. Randomly assign half of the mice to start with the “left” side and the other half with the “right” side. Pellets should be large enough so that they do not initially fall through the hopper bars

Day 1: Provide chow (~30g) in only the left or right hopper compartment.
Day 2: Move remaining chow to the other hopper.

3. Testing

Day 1: Place the treatment diet (~30g) in the assigned “left” or “right” starting compartment and normal chow in the other compartment (~30g) for 24 hours. *Note: The majority of published work using similar protocols will remove this first day of data from their analysis and consider it as time in which the mice became acclimated to the novel diet. HFD pellets tend to be flakey and should be gently compacted for weighing and before being placed into the hopper.*
Day 2: Switch mice to a new cage with clean bedding. Provide new treatment diet and normal chow, also swap their side positions for 24 hours. This is often considered the first day of testing.
Day 3: At the same time as Day 2, weigh the remaining diet. Search the cage for any pieces of diet that may have fallen through the hopper bars. This should be included in the remaining diet weight. Add new HFD or chow to bring total amounts back to ~30g, also swap their side positions for 24 hours.
Day 4: At the same time as Day 3, weigh the remaining diet. Search the cage for any pieces of diet that may have fallen through the hopper bars. This should be included in the remaining diet weight.

Notes:

- Ensure pellets are evenly distributed across hopper (see image)
- When mice consume normal chow out of a dish placed on the bottom of the cage (instead of the hopper), they can drop approximately 0-0.2g of powdered diet
    - Some studies housed mice on bars so they could collect spillage
    - Some studies said the amount was negligible

References and notes on methods used for protocol:

1. Buttigieg A, Flores O, Hernandez A, Saez-Briones P, Burgos H, and Morgan C (2014) Preference for hig-fat diet is developed by young Swiss CD1 mice after short-term feeding and is prevented by NMDA receptor antagonists. *Neurobiology of Learning and memory* 107: 13-18. Doi: 10.1016/j.nlm.2013.10.018

- Made food receptacles out of “a small glass placed in a bowl to collect spillage”
- Diet was placed at opposite corners of the cage and locations were switched every day to avoid side bias
- Food consumed was weight and replaced daily
- Mice were weight daily

2. Treesukosol Y, Sun B, Moghadam AA, Liang NC, Tamashiro KL, and Moran TH (2014) Maternal high-fat diet during pregnancy and lactation reduces the appetitve behavioral component in female offspring tested in a brief-access taste procedure. *Am J Physiol Integr Comp Physiol* 306: R499-R509

- Behavior began at least ~11wks of age
- Mice were given ad lib access to chow and HFD for 7 consecutive days
- Preference ratio calculation = (Intake of HFD) divided by (Intake of HFD + Intake of Chow)

3. Ong ZY and Muhlhausler BS (2011) Maternal "junk-food" feeding of rat dams alters food choices and development of mesolimbic reward pathway in the offspring. *FASEB J*. 25, 2167-2179, doi: 10.1096/fj.10-178392.

- Rat protocol comparing control and cafeteria junk-food diet ad libitum
- Pups were housed 3-4 rats/cage.
- Total intake calculated each week and divided by # of rats in cage
- Because of co-housing, calculated food intake was normalized to pup's body weight at end of each week

4. Vucetic Z, Kimmel J, Totoki K, Hollenbeck E, and Reyes TM (2010) Maternal high-fat diet alters methylation and gene expression of dopamine and opioid-related genes. *Endocrinology*. 151(10): 4756-4764, doi: 10.1210/en.2010-0505

- Offspring were caged individually during testing (age 24-26 wk)
- Day1 data was not analyzed because animals acclimated to the novel diet
- Day2 and 3 were averaged for analysis (24 hr)
- Intake of each diet was normalized to body weight
- Fat preference was calculated as % of HFD consumed in related to total food intake

5. Trillou CR, Delgorge C, Menet C, Arnone M, and Soubrie P (2004) CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity. *International Journal of Obesity* 28, 640-648. Doi: 10.1038/sj.ijo.0802583

- Offered normal chow and HFD in two separate hanging feeders
- Started testing on 9wk old mice
- Body weight was recorded daily for 11 weeks
- Food intake was monitored daily for the first 4 weeks and then week 12

6. Smith BK, Andrews K, and West DB (2000) Macronutrient diet selection in thirteen mouse strains. *Am J Physiol Regulatory Inegrative Comp Physiol* 278: R797-R805

- Behavior testing started at 7 wk of age
- Body weight was recorded twice per week
- Used powered diets and fat sources (corn-starch, powdered sugar, vegetable shortening…) were presented in custom glass jars
    - 2 oz glass jars
    - Stainless steel lid (diameter of lid hole measured 7/8 in)
    - Food all dry diets (carbs and protein), stainless steel discs, i.e. "food followers", were placed under the lid
        - These discs had 6 circular openings (each 7/16 in diameter) to allow food access while minimizing spillage

7. Kinney NE and Antill RW (1995) Role of Olfaction in the Formation of Preference for High-Fat Foods in Mice. *Physiology and Behavior* 59(3):475-478.

- During a 4day pretest period, all mice (before treatment, i.e. surgery) were given continuous access to both normal and HFD
- On day 5, all mice were food deprived for 12hr and then given a preference test for 2 hrs. Consumption was recorded for all mice
- Mice underwent treatment surgery, and on post-survey day7 (aka experimental day 13), mice were again food deprived for 12hrs and given a 2hr preference test
- Further preference testing was done again 15, 21, and 35 days post-surgery (they did this to test the return of olfactory function by day 21)

2018

ENVIGO

Teklad Global 18% Protein Rodent Diet

Product Description- 2018 is a fixed formula, non-autoclavable diet manufactured with high quality ingredients and designed to support gestation, lactation, and growth of rodents. 2018 does not contain alfalfa, thus lowering the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from 150 to 250 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fish meal minimizes the presence of nitrosamines. **Also available certified (2018C) and irradiated (2918). For autoclavable diet, refer to 2018S (Sterilizable) or 2018SX (Extruded & Sterilizable).**

Ingredients (in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, kaolin, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B_{12} supplement, folic acid, biotin, vitamin D_3 supplement, cobalt carbonate.

Standard Product Form: Pellet

Macronutrients		
Crude Protein	%	18.6
Fat (ether extract) [a]	%	6.2
Carbohydrate (available) [b]	%	44.2
Crude Fiber	%	3.5
Neutral Detergent Fiber [c]	%	14.7
Ash	%	5.3
Energy Density [d]	kcal/g (kJ/g)	3.1 (13.0)
Calories from Protein	%	24
Calories from Fat	%	18
Calories from Carbohydrate	%	58
Minerals		
Calcium	%	1.0
Phosphorus	%	0.7
Non-Phytate Phosphorus	%	0.4
Sodium	%	0.2
Potassium	%	0.6
Chloride	%	0.4
Magnesium	%	0.2
Zinc	mg/kg	70
Manganese	mg/kg	100
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	200
Selenium	mg/kg	0.23
Amino Acids		
Aspartic Acid	%	1.4
Glutamic Acid	%	3.4
Alanine	%	1.1
Glycine	%	0.8
Threonine	%	0.7
Proline	%	1.6
Serine	%	1.1
Leucine	%	1.8
Isoleucine	%	0.8
Valine	%	0.9
Phenylalanine	%	1.0
Tyrosine	%	0.6
Methionine	%	0.4
Cystine	%	0.3
Lysine	%	0.9
Histidine	%	0.4
Arginine	%	1.0
Tryptophan	%	0.2

Vitamins		
Vitamin A [e, f]	IU/g	15.0
Vitamin D_3 [e, g]	IU/g	1.5
Vitamin E	IU/kg	110
Vitamin K_3 (menadione)	mg/kg	50
Vitamin B_1 (thiamin)	mg/kg	17
Vitamin B_2 (riboflavin)	mg/kg	15
Niacin (nicotinic acid)	mg/kg	70
Vitamin B_6 (pyridoxine)	mg/kg	18
Pantothenic Acid	mg/kg	33
Vitamin B_{12} (cyanocobalamin)	mg/kg	0.08
Biotin	mg/kg	0.40
Folate	mg/kg	4
Choline	mg/kg	1200
Fatty Acids		
C16:0 Palmitic	%	0.7
C18:0 Stearic	%	0.2
C18:1ω9 Oleic	%	1.2
C18:2ω6 Linoleic	%	3.1
C18:3ω3 Linolenic	%	0.3
Total Saturated	%	0.9
Total Monounsaturated	%	1.3
Total Polyunsaturated	%	3.4
Other		
Cholesterol	mg/kg	--

[a] Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

[b] Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

[c] Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

[d] Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

[e] Indicates added amount but does not account for contribution from other ingredients.

[f] 1 IU vitamin A = 0.3 µg retinol

[g] 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

Teklad Diets are designed and manufactured for research purposes only.

0915

High-Fat Diet Data Sheet from Supplier

Teklad Custom Research Diet Data Sheet

TD.03584 35% Lard Diet (Adj., No C)

Formula	g/Kg
Casein	230.0
DL-Methionine	3.5
Sucrose	150.0
Maltodextrin	191.35
Lard	350.0
Mineral Mix, AIN-93G-MX (94046)	50.0
Calcium Phosphate, monobasic, monohydrate	2.5
Vitamin Mix, AIN-93-VX (94047)	18.4
Choline Bitartrate	4.25

Footnote

A diet used to induce obesity. A modification of TD.03307, using a vitamin mix without vitamin C. Approximate fatty acid profile (% total fat): 40% saturated, 50% monounsaturated, 10% polyunsaturated. Cited in Circ Res (2005) 96:1178-1184.

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	20.4	15.0
Carbohydrate	36.1	26.6
Fat	35.2	58.4
Kcal/g	**5.4**	

[1] Values are calculated from ingredient analysis or manufacturer data

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Speak With A Nutritionist

- **(800) 483·5523**
- **askanutritionist@harlan.com**

Harlan Laboratories · PO Box 44220 · Madison, WI 53744-4220
www.harlan.com

Key Features

- **Purified Diet**
- **Diet Induced Obesity**
- **Lard**
- **Dough Form or Pellets**

Key Planning Information

- *Products are made fresh to order*
- Store product at 4°C or lower
- Use within 6 months (applicable to most diets)
- Box labeled with product name, manufacturing date, and lot number
- Replace diet at minimum once per week
  More frequent replacement may be advised
- Lead time:
  · 2 weeks non-irradiated
  · 4 weeks irradiated

Product Specific Information

- 1/2" Pellet or Powder (crumbly)
- Minimum order 3 Kg
- Irradiation available upon request

Options (Fees Will Apply)

- Rush order (pending availability)
- Irradiation (see Product Specific Information)
- Vacuum packaging (1 and 2 Kg)

International Inquiry

·*Outside U.S.A. or Canada*·

- **askanutritionist@harlan.com**

Place Your Order (U.S.A. & Canada)

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- **(800) 483·5523**
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- **tekladinfo@harlan.com**

Helping you do research better

02/17/09 BDM

Calculate sucrose and sucralose treatments for mouse from human equivalent dose (HED)

- According the NHANES (Marriott et al 2010) the average person consumes 83 grams of sugar, which is 3.458 times the recommended daily maximum.
- Dose should not simply be extrapolated using body weight
- It is essential to appropriately translate dosage from one species to another
- FDA recommends extrapolating using the Body Surface Area (BSA)
- Given
  - Mouse Km = 3
  - Human Km = 37 (assumes human is 60 kg)

$$HED\ \left(\frac{mg}{kg}\right) = \ Animal\ dose\ \left(\frac{mg}{kg}\right) \cdot \frac{Animal\ Km}{Human\ Km}$$

Sucralose ($C_{12}H_{19}Cl_3O_8$) calculation

- The HED sucralose dose (a.k.a. FDA ADI guidelines) is 5 mg/kg x 3.458 = 17.29 mg/kg

$$17.29\frac{mg}{kg}\ HED = ?\frac{mg}{kg} \cdot \left(\frac{3}{37}\right) \rightarrow\ 213.24\frac{mg}{kg}\ sucralose\ for\ a\ mouse$$

- The daily treatment of a 25 g mouse would be 1.54 mg sucralose x 3.458 = 5.33 mg/kg
- Calculate the sucralose concentration given
  - sucralose MW of 397.76 g/mol
  - a daily sucralose treatment using 2 mL H_2O

$$5.33\ mg\ sucralose \cdot \frac{1g}{1000mg} \cdot \frac{mol}{397.67g} \cdot \frac{1000mmol}{1mol} \cdot \frac{}{.002\ L} = \mathbf{6.7\ mM\ sucralose}$$

- On a typical sucralose Lickometer curve from our lab, this concentration produces a positive response at ~40% up the curve (slightly below the EC50)

Sucrose ($C_{12}H_{22}O_{11}$) calculation

- The sucrose HED (a.k.a. ADA recommendation maximum) is 400 mg/kg
  - More specially, 24 grams daily for females (assume weight is 60 kg)

$$1383\frac{mg}{kg}\ HED = ?\frac{mg}{kg}\ x\ \left(\frac{3}{37}\right) \rightarrow 17{,}061\frac{mg}{kg}\ sucrose\ for\ a\ mouse$$

- For an average 25 g mouse the daily treatment would be 123.3 mg sucrose x 3.458 = 426.5 mg/kg
- Calculate the sucrose concentration given

- sucrose MW of 342.2965 g/mol
- a daily sucrose treatment using 2 mL H_2O

$$426.5\ mg\ sucrose \cdot \frac{1g}{1000mg} \cdot \frac{mol}{342.2965g} \cdot \frac{}{.002\ L} = .\mathbf{623\ M\ sucrose}$$

- On a typical sucrose Lickometer curve from our lab, this concentration produces a positive response at ~40% up the curve (slightly below the EC50)

References

- *Guidance for Industry - Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*
- Reagan-Shaw, S., Nihal, M., and Ahmad, N. Dose translation from animal to human studies revisited *FASEB J* March 2008 22:659-661; published ahead of print October 17, 2007, doi:10.1096/fj.07-9574LSF
- Marriott BP, Olsho L, Hadden L, Connor P (2010) Intake of Added Sugars and Selected Nutrients in the United States, National Health and Nutrition Examination Survey (NHANES) 2003-2006. Crit Rev Food Sci Nutri 50:228-258.